From the Department of Medical Biochemistry and Biophysics
Karolinska Institutet, Stockholm, Sweden

GENE EXPRESSION IN BUCCAL KERATINOCYTES WITH EMPHASIS ON CARBONYL METABOLISM

Claudia A. Staab

Karolinska Institutet

Stockholm 2008

Published by Karolinska Institutet.

ISBN 978-91-7409-127-4

Printed by

www.reproprint.se
Gårdsvägen 4, 169 70 Solna

Une sortie, c'est une entrée que l'on prend dans l'autre sens.

Boris Vian

ABSTRACT

The inner lining of the cheek, the buccal mucosa, is a target for air-borne, dietary and tobacco usage-derived carcinogens, but also interesting from a drug delivery point of view. Cancer arising in the buccal epithelium, buccal squamous cell carcinoma (BSCC), often diagnosed at a late disease stage, is highly aggressive and recurrent, emphasizing the need for novel approaches in diagnosis and therapy.

An *in vitro* model for human buccal carcinogenesis consisting of normal buccal keratinocytes (NBK) and two transformed cell lines of buccal origin was applied to explore mechanisms of buccal carcinogenesis, tumor marker and drug target expression. Two-dimensional gel electrophoresis, DNA microarray analysis, and the application of three bioinformatics programs for data mining allowed for the identification of multiple established and potential novel markers for BSCC, including tumor promoter/suppressor genes. Furthermore, post-confluent culture of NBK in absence and presence of fetal bovine serum was successfully used to induce terminal squamous differentiation (TSD) to various extents and thus enrich for different *strata* of the epithelium. Here, expression and activity of carbonyl-metabolizing enzymes (CMEs) were assessed in view of their multiple roles in phase I biotransformation. The combined results put forward *ADH3* transcription as a proliferation marker as well as *ALDH4A1*, *AKR1A1* and *HPGD* transcription as markers for the onset of TSD. Finally, the findings suggested increased CME-mediated xenobiotic activity at the onset of TSD, with potential implications for drug response and BSCC susceptibility.

In addition to its function as a ubiquitous formaldehyde scavenger, including in the buccal mucosa, alcohol dehydrogenase 3 (ADH3) irreversibly reduces S-nitroso-glutathione (GSNO) which implies an important role in nitric oxide homeostasis. Assessment of ADH3 in terms of formaldehyde/GSNO competition provided indirect evidence for formaldehyde as a physiological trigger of ADH3-mediated GSNO depletion with possible direct implications for asthma, where formaldehyde is known for exacerbating and GSNO for protective effects. Moreover, GSNO reduction resulted in the glutathione-controlled formation of glutathione transferase inhibitors, thus possibly affecting phase II biotransformation under conditions of oxidative stress. In light of these ADH3-mediated adverse effects, several substrate analogues were tested as ADH3 inhibitors. The results provide guidelines for future design of a specific ADH3 inhibitor with potential direct clinical use for the prevention of asthma-exacerbating effects.

LIST OF PUBLICATIONS

This thesis is based on the following articles, referred to in the text by their Roman numerals.

I. **Staab C.A.**, Ceder R., Jägerbrink T., Nilsson J.A., Roberg K., Jörnvall H., Höög J.O. and Grafström R.C. (2007) Bioinformatics processing of protein and transcript profiles of normal and transformed cell lines indicates functional impairment of transcriptional regulators in buccal carcinoma. *J. Proteome Res.* **6**(9):3705-3717.

II. Nilsson J.A., Hedberg J.J., Vondracek M., **Staab C.A.**, Hansson A., Höög J.O. and Grafström R.C. (2004) Alcohol dehydrogenase 3 transcription associates with proliferation of human oral keratinocytes. *Cell. Mol. Life Sci.* **61**(5):610-617.

III. **Staab C.A.**, Ceder, R., Roberg, K., Grafström, R.C. and Höög, J.O. (2008) Serum-responsive gene expression of carbonyl-metabolizing enzymes in cultured human buccal keratinocytes. *Cell. Mol. Life Sci.*, in press.

IV. **Staab C.A.**, Ålander J., Brandt M., Lengqvist J., Morgenstern R., Grafström R.C. and Höög J.O. (2008) Reduction of S-nitrosoglutathione by alcohol dehydrogenase 3 is facilitated by substrate alcohols via direct cofactor recycling and results in GSH-controlled formation of glutathione transferase inhibitors. *Biochem. J.*, **413**(3):493-504.

V. **Staab C.A.**, Hellgren M., Grafström R.C. and Höög J.O. Medium-chain fatty acids and glutathione derivatives as inhibitors of alcohol dehydrogenase 3-mediated GSNO reduction, submitted.

RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

1. **Staab C.A.**, Vondracek M., Custodio H., Johansson K., Nilsson J.A., Morgan P., Höög J.O., Cotgreave I. and Grafström R.C. (2004) Modelling of normal and premalignant oral tissue by using the immortalised cell line, SVpgC2a: a review of the value of the model. Review. *Altern. Lab. Anim.* **32**(4):401-405.

2. Höög J.O., **Staab C.A.**, Hedberg J. and Grafström R.C. (2006) Mammalian alcohol dehydrogenase 3 (ADH3) has several essential functions. In: Enzymology and Molecular Biology of Carbonyl Metabolism - 12 (Weiner H., Plapp B., Lindahl R., Maser E., eds.), pp. 154-160, Purdue University Press, Indiana.

3. Ceder R., Merne M., **Staab C.A.**, Nilsson J.A., Höög J.O., Dressler D., Engelhart K. and Grafström R.C. (2007) The application of normal, SV40 T-antigen-immortalised and tumour-derived oral keratinocytes, under serum-free conditions, to the study of the probability of cancer progression as a result of environmental exposure to chemicals. *Altern. Lab. Anim.* **35**(6):621-639.

4. **Staab C.A.**, Hellgren M. and Höög J.O. (2008) Dual functions of alcohol dehydrogenase 3 – implications with focus on formaldehyde dehydrogenase and S-nitrosoglutathione reductase activities. Review. *Cell. Mol. Life Sci.*, in press.

5. **Staab C.A.**, Ålander J., Morgenstern R., Grafström R.C. and Höög J.O. The Janus face of alcohol dehydrogenase 3. *Chem.-Biol. Interact.* (Special Issue: Enzymology and Molecular Biology of Carbonyl Metabolism), in press.

CONTENTS

LIST OF ABBREVIATIONS

ACAT1	Acetyl-CoA acetyltransferase 1
ADH	Alcohol dehydrogenase
AKR	Aldo-keto reductase
ALDH	Aldehyde dehydrogenase
BSCC	Buccal squamous cell carcinoma
CBR	Carbonyl reductase
CDC2	Cell division control protein 2
CFE	Colony-forming efficiency
CK	Cytokeratin
CME	Carbonyl-metabolizing enzyme
CYP	Cytochrome P450
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
EGF	Epidermal growth factor
ESI MS	Electrospray ionization mass spectrometry
ESTD	Esterase D
FBS	Fetal bovine serum
GO	Gene Ontology
GOTM	Gene Ontology Tree Machine
GSH	Glutathione
GSNO	S-nitrosoglutathione
GSSG	Glutathione disulfide
GST	Glutathione S-transferase
HMGSH	S-Hydroxymethylglutathione
hnRNP	Heterogeneous nuclear ribonucleoprotein
HPGD	15-(NAD^+)-hydroxyprostaglandin dehydrogenase
HPV	Human papillomavirus
HSD11B1	11-β-hydroxysteroid dehydrogenase type I
HSP	Heat shock protein
IPA	Ingenuity pathway analysis
IVL	Involucrin
MALDI-TOF MS	Matrix-assisted laser desorption/ionization - time-of-flight mass spectrometry
MDR	Medium-chain dehydrogenases/reductases
NBK	Normal buccal keratinocytes
NNK	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
NO	Nitric oxide
NQO	NAD(P)H:quinone acceptor oxidoreductase
PAH	Polycyclic aromatic hydrocarbon
PCNA	Proliferating cell nuclear antigen
QOR	Quinone oxidoreductase
SCC	Squamous cell carcinoma
SDR	Short-chain dehydrogenases/reductases
SFN	Stratifin
SNO	S-nitrosothiol

SPR	Small proline-rich protein
StaRT-PCR	Standardized reverse transcriptase-polymerase chain reaction
SV40	Simian Virus 40
SV40T	Simian Virus 40 large T antigen
TGF	Transforming growth factor
TGM	Transglutaminase
TSD	Terminal squamous differentiation

Three and one letter codes for the 20 naturally occurring amino acids

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Iso	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

1 INTRODUCTION

The inner lining of the cheek, the buccal mucosa, is continuously exposed to inhalatory and dietary carcinogens. Cancer arising in the buccal epithelium, buccal squamous cell carcinoma (BSCC), belongs to the most aggressive and recurrent oral cancer types [1, 2]. Despite advances in clinical assessment and treatment methods, survival rates have not improved, mostly due to diagnosis at late disease stages and cancer therapy resistance [3-5]. Hence, there is a clear need for novel approaches in diagnosis and therapy.

In order to develop and assess new strategies, robust and reproducible test model systems for buccal carcinogenesis are necessary. To this end, the present thesis involved the use of an *in vitro* model of buccal carcinogenesis consisting of normal buccal keratinocytes (NBK) and two transformed buccal keratinocyte lines termed SVpgC2a and SqCC/Y1, which show characteristics of premalignancy and full-blown malignancy, respectively [6-10]. Furthermore, standardized serum-free culture conditions established for NBK allow for the use of fetal bovine serum (FBS) as inducer of terminal squamous differentiation (TSD), the major pathway of programmed cell death in the buccal epithelium [11, 12]. This permits the culture of differentiated, suprabasal-like NBK in addition to highly proliferative, basal-like NBK. Notably, SVpgC2a and SqCC/Y1 can be cultured under identical conditions, but have acquired TSD-deficiency in the process of immortalization/malignant transformation [7, 12].

In this context, the characterization of xenobiotic metabolism is of particular interest for several reasons. Obviously, elucidation of detoxification and carcinogen activation mechanisms in the normal buccal mucosa can help us understand susceptibility to BSCC. Additionally, knowledge about xenobiotic metabolism in the buccal mucosa is important for its use as drug delivery site [13, 14]. Moreover, xenobiotic enzymes that exhibit altered expression in a (pre-)malignant tissue state might qualify as drug targets, with the use of specifically designed drugs that can either be neutralized in normal cells or activated in (pre-)malignant cells. Finally, transformed cell lines, when used as models for the respective normal tissue in toxicological studies, generally need to be evaluated in terms of metabolic competence which can differ considerably from the cell type of origin [15].

Carbonyl metabolizing enzymes (CMEs), *i.e.* enzymes that are involved in the metabolism of carbonyl-group containing compounds, play an important role in human xenobiotic metabolism [16]. CMEs convert exogenous substrates that are highly relevant for normal as well as neoplastic buccal epithelium, as they contribute to the metabolism of oral cancer risk factors, *e.g.* ethanol, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and polycyclic aromatic hydrocarbons (PAH), as well as anti-cancer therapeutics, *e.g.* retinoids and anthracyclines [17-23]. The simplest carbonyl compound, formaldehyde, has recently been classified as a carcinogen [24]. Among all CMEs, alcohol dehydrogenase 3 (ADH3) is the most efficient and wide-spread formaldehyde scavenger in human tissues including the buccal mucosa and the described *in vitro* model systems thereof [25-27]. During the last decade, evidence has accumulated that suggests an additional important role for ADH3 in endogenous nitric oxide (NO) homeostasis [28-32]. In fact, many CMEs are equally involved in exogenous and endogenous metabolism, but few have been investigated in terms of exogenous/endogenous substrate competition, a subject of great toxicological and pharmacological interest [33].

Within this thesis work, gene expression was assessed in the described *in vitro* model for buccal carcinogenesis and evaluated using bioinformatics tools with the long-term aim to elucidate mechanisms of carcinogenesis, find novel tumor markers and, finally, propose novel diagnostic strategies. The studies included a global expression assessment (Paper I) as well as two targeted approaches for the investigation of CME expression, including ADH3 transcription, in proliferative and differentiated buccal keratinocytes (Papers II and III). Finally, two studies deal with the enzyme ADH3 and implications of its dual function as formaldehyde dehydrogenase and S-nitrosoglutathione (GSNO) reductase (Papers IV and V).

2 BACKGROUND

2.1 THE BUCCAL MUCOSA

As major portal of entry, the oral cavity is an evident target for air-borne, dietary and tobacco usage-derived carcinogens. The inner linings of the oral cavity can be divided into three major categories: 1) Keratinizing mucosae, including the masticatory mucosae of the gingiva and the hard palate; 2) non-keratinizing mucosae, including the buccal mucosa, the floor of the mouth, and the mucosa of the esophagus; 3) the dorsum of the tongue, which is covered by a specialized mucosa [34]. Non-keratinizing mucosae as the buccal lining exhibit greater permeability than keratinizing mucosae, which is attributed to differences in intercellular lipid rather than degree of keratinization [13]. This property makes the buccal epithelium and the underlying tissue particularly vulnerable for carcinogenic compounds, but has also lead to a growing interest in the buccal mucosa as a drug delivery site [14].

2.1.1 Structure of the buccal mucosa

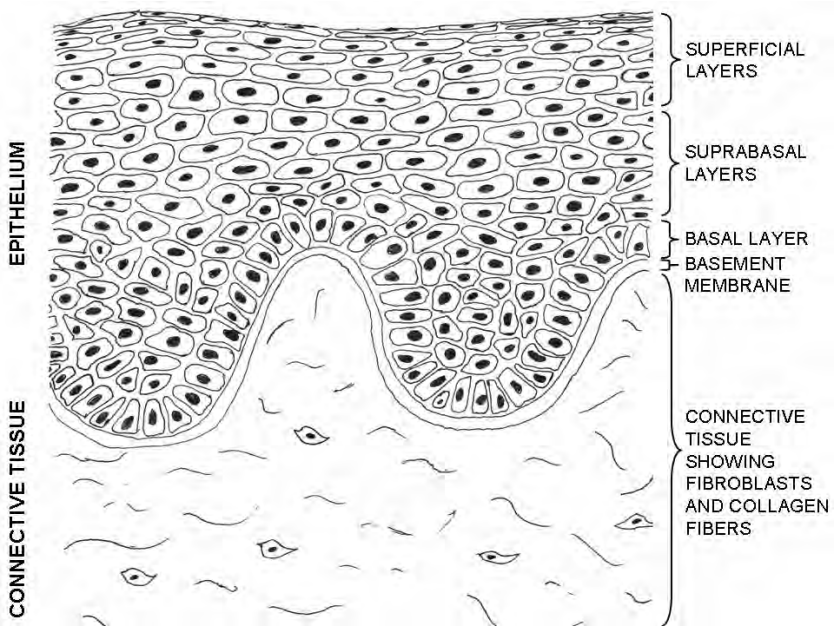


Fig. 1. Structure of the buccal mucosa, modified from Harris and Robinson [35].

The buccal mucosa is constituted of a stratified squamous epithelium supported by a fibrous connective tissue, more specifically called the *lamina propria mucosae* (Fig. 1). These two tissue compartments are separated by the basement membrane, an extracellular matrix constituted of a network of collagens, laminins and other proteins [36]. The present thesis deals with cultured cells of the buccal epithelium. As all stratified squamous epithelia, this epithelium serves to protect the underlying tissue from mechanical, physical, chemical and microbial insult and therefore must undergo constant repair and renewal [37]. The different keratinocyte layers can be subdivided into three parts, the basal layer, the suprabasal layers and the superficial layers (Fig. 1) [14, 34, 38]. The innermost basal layer harbors amplifying cells and a small proportion of infrequently dividing stem cells, which together form the basis of the proliferative potential of the tissue. Normal tissue homeostasis is dependent on a fine-tuned balance between proliferation and a well-defined terminal differentiation program called terminal squamous differentiation (TSD) [34].

2.1.2 Terminal squamous differentiation (TSD)

TSD is the major pathway of cell death in all stratified epithelia. As keratinocytes mature, they migrate from basal to suprabasal and superficial layers, while they gradually lose their proliferative ability and undergo TSD until finally dead cells are shed off from the outmost layer [34]. In keratinizing epithelia, TSD leads to the production of the outmost *stratum corneum*, a layer of flat cornified cells densely filled with cytokeratin (CK) filaments and surrounded by a thickened cell envelope. The process of TSD in non-keratinizing epithelia including the buccal subsite is also accompanied by morphological changes, albeit of less distinct character (Fig. 2) [34]. As cells differentiate, they gradually enlarge, flatten and form a cross-linked protein envelope, a modified or "incomplete" version of the tougher cornified envelope found in keratinizing epithelia. Precursors or inactive forms of protein envelope components are already expressed in suprabasal cells while the assembly mainly takes place in the surface cells. Also the composition of CKs, the predominant cytoskeletal proteins in all kinds of epithelia, changes during the process of TSD. At the same time, expression of proteins that associate with proliferation is gradually downregulated [34, 37, 38]. The median turnover time of the buccal epithelium is 14 days [34]. Various proteins differentially expressed during buccal TSD and their functions are given in Table 1; their approximate distribution in the buccal epithelium is illustrated in Fig. 2.

Table 1: Proteins differentially expressed during TSD [37-41].

Protein (Abbr.)	Gene	Cellular component	Function
Cytokeratin 5 (CK5)	*KRT5*	Cytoskeleton	Components of intermediate filaments
Cytokeratin 14 (CK14)	*KRT14*		
Cytokeratin 4 (CK4)	*KRT4*		
Cytokeratin 13 (CK13)	*KRT13*		
Cytokeratin 19 (CK19)	*KRT19*		
Involucrin (IVL)	*IVL*	Crosslinked protein envelope	Components of the crosslinked protein envelope
Small proline-rich protein 1 (SPR1)	*SPRR1*		
Small proline-rich protein 3 (SPR3)	*SPRR3*		
Transglutaminase 1 (TGM1)	*TGM1*		Crosslinking of components of the protein envelope

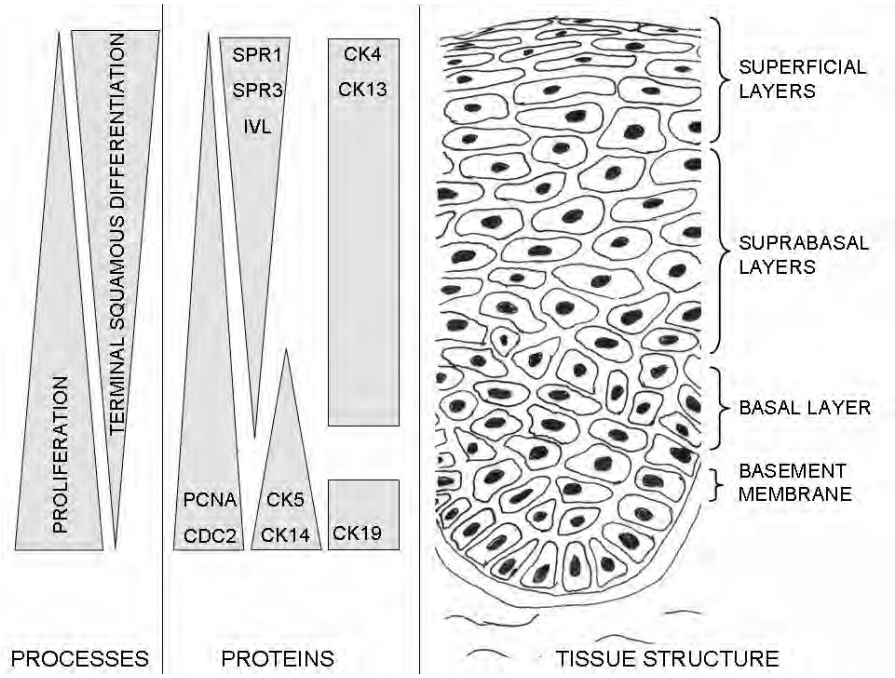


Fig. 2. Structure of the buccal epithelium. Ongoing processes and proteins associated with different layers are indicated. Protein abbreviations are as in Table 1. PCNA, Proliferating cell nuclear antigen; CDC2, Cell division control protein 2

Regulation of tissue homeostasis in stratified epithelia relies on the action of various cytokines including such that stimulate proliferation, *e.g.* epidermal growth factor

(EGF) and transforming growth factor α (TGF-α), as well as such that induce TSD, *e.g.* TGF-β and interferon-γ [11, 34, 42]. Furthermore, a range of low-molecular compounds induce or modulate keratinocyte differentiation, *e.g.* retinoids, triiodothyronine, vitamin D_3 and glucocorticoids [43-46]. Finally, the presence of a calcium gradient in stratified epithelia *in vivo* suggests the involvement of calcium in the induction or regulation of TSD [47]. Indeed, calcium is often used as inducer of epidermal differentiation *in vitro* and can equally induce certain, notably not all, characteristics of TSD in cultured buccal keratinocytes [9, 42].

2.1.3 Buccal squamous cell carcinoma (BSCC)

Oral cavity cancer is the 8th most common cancer type world-wide and thus a major global health problem [48]. More than 95 % of all mouth neoplasms are squamous cell carcinomas (SCCs), meaning that they arise in epithelia [49-51]. BSCC is characterized by a high tendency of locoregional recurrence and poor survival rates and thus considered one of the most aggressive oral cancer types [1, 2, 52]. Although relatively rare in most Western countries, BSCC belongs to the most common cancer forms in South and Southeast Asia [48, 51, 53]. This is in large due to the prevalence of betel quid chewing, which undoubtedly represents one of the major risk factors for BSCC [53-56]. Other important risk factors include tobacco chewing and smoking, alcohol abuse, dietary deficiencies and viruses, particularly human papillomavirus (HPV) [55-58].

Currently, diagnosis of oral (pre-)malignant lesions still mostly relies on visual inspection including clinical staging and microscopic assessment of tissue specimens and cells [3, 56, 59, 60]. The prognostic value of molecular markers is subject of extensive current research, but so far neither an individual marker nor a panel of markers has been determined that is more reliable than the above mentioned conventional methods [3, 61, 62]. However, the establishment of laser-capture microdissection, array technologies and other -omics techniques for diagnostic tumor profiling is being pursued and there is no doubt that molecular markers have a strong potential in future oral cancer prognosis as well as individualized therapy [63-65].

Retinoids, β-carotene, α-tocopherol and ascorbic acid have been used in chemopreventive studies of oral premalignant lesions, *i.e.* in efforts to suppress malignant transformation and reverse lesions to a normal state, albeit with inconsistent

outcome [4, 66, 67]. Oral SCCs are mainly treated by surgical resection, radiotherapy, chemotherapy, or a combination thereof. Novel treatment strategies are being developed and evaluated, including targeted molecular therapies, *e.g.* antibodies against overexpressed tumor promoters and gene transfer of tumor suppressor genes [66, 68-70].

2.1.4 Oral carcinogenesis

Few studies with an emphasis on human buccal carcinogenesis have been performed. Carcinogenesis of the buccal subsite has largely been studied in the carcinogen-induced hamster cheek pouch carcinogenesis model [71]. However, the hamster cheek pouch epithelium is considerably different from its human counterpart, both regarding morphology and carcinogen susceptibility, which complicates inter-species extrapolation and emphasizes the need for suitable human models [72].

Generally, oral carcinogenesis is a combined result of an individual's genetic predisposition and exposure to carcinogens and considered to be a multistep process (Fig. 3). First, a cell undergoes initiation by an initial genetic insult affecting a central tumor promoting or suppressor gene, *e.g.* a mutation, chromosomal insertion/deletion or change in DNA ploidy. Frequently overexpressed proteins in oral SCCs include such that mediate self-sufficiency in growth signals, *e.g.* EGF receptor and transcription factors of the Myc family [61-63, 70]. On the contrary, inactivation of tumor suppressor genes leads to acquisition of insensitivity to anti-growth signals and evasion of apoptosis. In oral SCCs, *RB1* (encoding retinoblastoma-associated protein, Rb), *TP53* (encoding p53) and *CDKN2A* (encoding p16) are among the loci that are most often altered, mutated or lost [61, 63, 70]. Initiation is followed by promotion involving the formation of a cluster of preneoplastic cells, a so-called premalignant lesion, which is in principle still reversible. The acquisition of further genetic alterations and increasing genetic instability leads to conversion to a malignant phenotype and finally progression into metastatic cancer [70, 72].

Also viral infection can be the cause of genetic alterations in such key regulator genes. Multiple lines of evidence suggest that mucosal HPV is involved in the etiology of oral SCC [73]. Its oncogenic properties are mainly attributed to the actions of two viral proteins, E6 and E7, which among others inhibit activities of p53 and Rb, respectively [58, 74]. Studies on the involvement of other viruses, *e.g.* Epstein-Barr

virus and hepatitis C virus have provided inconclusive results [75]. Notably, simian virus 40 (SV40), originally discovered about 50 years ago as a contaminant in polio vaccine stocks, expresses SV40 large T antigen (SV40T), a protein that binds and inactivates p53 and Rb, similarly to the E6/E7 proteins of HPV [76]. This property is sufficient to immortalize epithelial cells *in vitro* [77]. However, a causal role of SV40 in human cancers is still under debate [76].

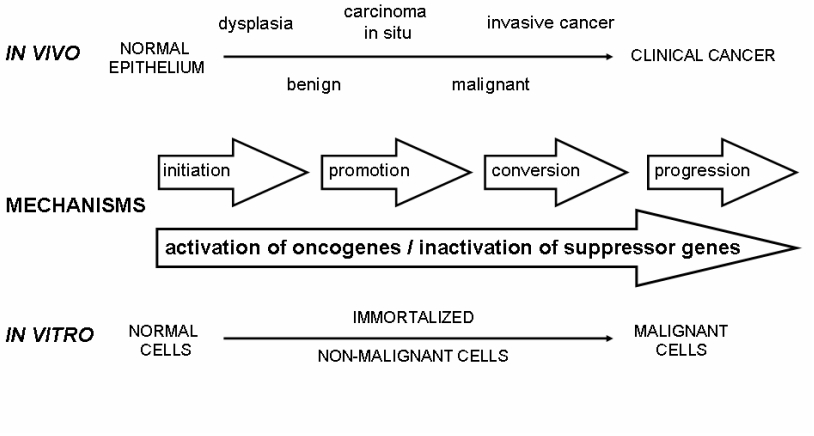


Fig. 3. Oral carcinogenesis is a multistep process [72].

2.1.5 *In vitro* models for the oral mucosa

In vitro models for normal and transformed states of the oral mucosa are widely used for studies of toxicology, carcinogenesis mechanisms and chemoprevention. Notably, there are only very few cell culture models for the buccal mucosa [72, 78]. In principle, the use of normal oral keratinocytes would be the optimal choice for any kind of *in vitro* study on normal keratinocyte biology including toxicity testing. But all normal oral keratinocytes exhibit a finite life span due to the fact that they have retained the ability to undergo TSD in culture, which results in a natural limit in cell numbers. Hence, investigators often resort to transformed cell lines instead, including experimentally immortalized cell lines or such derived from (pre)malignant lesions, as they can be relatively effortlessly, reproducibly and limitlessly cultured [79]. However, besides the gained ability to circumvent normal cell death, the immortalization process also often results in metabolic deficiencies or alterations which make the extrapolation to normal cells difficult [15]. Thus, if used for toxicology testing, continuous cell lines should optimally be evaluated in terms of relevant metabolic competence.

Immortal oral keratinocyte lines have been established from dysplastic human oral mucosa as well as by infection with oncogenic viral antigens from HPV or SV40. As such cell lines often exhibit a premalignant-like, yet non-tumorigenic, phenotype, they can be good model systems to study chemoprevention or malignant transformation [8, 72, 78, 80]. Notably, the SV40T-immortalized cell line used within this thesis work, SVpgC2a (described in more detail below), is the only existent immortalized human cell line from the buccal subsite. Numerous tumor cell lines have been derived from various oral SCCs including a few from BSCC. These cell lines are commonly tumorigenic when injected into immunodeficient mice and thus can be considered models for full-blown malignancy [72, 78, 79]. The buccal tumor cell line SqCC/Y1, used within this thesis work, will be discussed in more detail below [6, 9].

Hence, a combination of normal oral keratinocytes, premalignant-like and tumor-derived cell lines has the potential to qualify for studies of oral cancer progression (cf. Fig. 3). In this context, however, a frequent impediment is the fact that immortalized/premalignant and malignant oral tumor cell lines are usually established and cultured under addition of FBS to the culture medium [72, 78, 79]. In contrast, normal oral keratinocytes are nowadays commonly cultured under serum-free conditions. These discrepancies in culture medium composition complicate *in vitro* studies on carcinogenesis substantially.

2.1.6 NBK, SVpgC2a and SqCC/Y1

Monolayer cultures of NBK can be grown from buccal biopsies obtained by maxillofacial surgery. Standardized serum-free conditions have several advantages: First, they omit the problems associated with FBS batch variations which improves reproducibility [12, 72]. Furthermore, FBS is a very efficient inducer of TSD, which probably reflects the involvement of multiple agents, as discussed above [9, 11, 42, 46, 72, 81]. Hence, omitting serum from the culture conditions helps NBK maintain a highly proliferative phenotype, which improves final cell yield [11, 72]. Importantly, the same standardized serum-free culture conditions used for NBK are applicable to SVpgC2a and SqCC/Y1. The morphologies of the three cell types cultured under serum-free conditions are presented in Fig. 4 and their properties are summarized in Table 2.

The cell line SVpgC2a was obtained by transformation of NBK (non-smoking 17-year-old donor) with a plasmid carrying the SV40T gene. The SV40T gene was integrated in the genome, stably expressed and the protein formed complexes with the wild-type p53 protein [77]. The resulting cell line was apparently immortal, exhibited a highly proliferative phenotype and yet was non-tumorigenic when injected into immunodeficient mice [77, 82]. SVpgC2a is TSD-deficient, *i.e.* shows decreased responsiveness to TSD-inducing agents including decreased growth inhibition and expression of markers of TSD. Furthermore, SVpgC2a displays a hypodiploid karyotype which is relatively stable throughout prolonged culture [83]. Finally, the keratin profile of SVpgC2a reminds of severe epithelial dysplasia, a typical feature of a premalignant lesion [10].

The cell line SqCC/Y1 is originally derived from a verrucous squamous cell carcinoma of a female patient and was established under conditions using serum, but later adapted to the serum-free culture conditions used for NBK [9]. The single p53 allele carries two missense mutations and is not expressed [6]. SqCC/Y1 is immortal, produces tumors when injected into immunodeficient mice, is resistant to induction of growth-arrest/TSD and shows decreased expression of TSD markers in response to TSD-inducing agents. Furthermore, with 63-83 chromosomes/metaphase SqCC/Y1 displays an aneuploid karyotype [9].

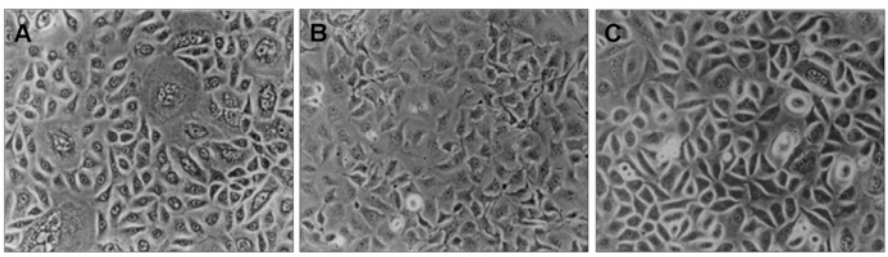


Fig. 4. Morphology of monolayer cultures of human buccal keratinocytes. (**A**) NBK show typical cobble-stone-like epithelial morphology. Some terminally differentiated cells with enlarged cell surface are present. (**B**) SVpgC2a show elongated fibroblast-like morphology. (**C**) SqCC/Y1 show cells of variable size and polygonal cell shape.

Table 2: Properties of the three cell types used within this thesis work [6-9, 12, 77].

Property	NBK	SVpgC2a	SqCC/Y1
Establishment	derived from biopsies following maxillofacial reconstructural surgery	derived from SV40T-transfection of NBK	derived from a buccal squamous cell carcinoma
Longevity	cultured for ≈ 3-4 weeks until 80% confluency after 2nd passage	immortal, > 1000 population doublings	immortal, > 1000 population doublings
p53 status	wild-type	wild-type p53 expressed but inactivated by SV40T	one single p53 allele with two missense mutations
Tumorigenicity	non-tumorigenic	non-tumorigenic	tumorigenic
TSD	undergoes TSD in culture	TSD-deficient	TSD-deficient
Karyotype	normal	hypodiploid (39-45 chromosomes/metaphase)	aneuploid (63-83 chromosomes/metaphase)

2.2 XENOBIOTIC METABOLISM

Xenobiotic metabolism describes the metabolism of exogenous compounds that are foreign to an organism's normal biochemistry, *i.e.* drugs and toxins that are not part of the normal diet. Absorption of most foreign compounds into the human body is by passive diffusion through the lipid bilayer of cell membranes; hence, most potentially toxic compounds are lipid soluble. Lipophilic compounds are readily reabsorbed after biliary excretion which complicates and delays their final elimination. In order to facilitate excretion, such compounds need to be converted to more hydrophilic compounds, a task which is accomplished by a set of metabolic pathways collectively referred to as biotransformation.

2.2.1 Reactions and enzymes in xenobiotic metabolism

Biotransformation is divided into three phases and involves a large variety of reactions and enzymes. Phase I reactions aim at introducing functional groups into

xenobiotic compounds which enables subsequent phase II reactions, *i.e.* conjugation reactions with hydrophilic compounds. Oxidations and reductions are among the most important types of phase I reactions. The majority of xenobiotic oxidations are executed by microsomal mono-oxygenases including the cytochrome P450 (CYP) system and the flavin-containing mono-oxygenases. Reductions can be catalyzed by both microsomal and cytosolic enzymes. For instance, cytotoxic quinones can be reduced by microsomal NADPH cytochrome P-450 reductase, but also by several cytosolic enzymes including carbonyl reductase 1 (CBR1) and NAD(P)H:quinone acceptor oxidoreductases (NQOs) [84-86]. The latter belongs to the group of xenobiotic carbonyl-metabolizing enzymes (CMEs) which will be discussed in more detail below [16]. A phase II reaction consists in the conjugation of the exogenous compound with an endogenous hydrophilic compound. For example, conjugation with glutathione (GSH) is catalyzed by glutathione transferases (GSTs), again involving both microsomal and cytosolic enzymes. Phase III reactions refer to the processing of a GSH conjugate as *e.g.* in the mercapturate pathway which yields the N-acetyl-cysteinyl conjugate (= mercapturate) as final product [87].

Notably, biotransformation does not necessarily lead to the detoxification, *i.e.* disposal of a foreign compound by excretion, but can actually underlie the toxicity of a compound by producing a chemically reactive metabolite. This process known as metabolic activation or bioactivation can be taken advantage of for cancer therapy with the design of tumor-activated prodrugs [88]. There often exist competitive metabolic pathways for a compound, some of which may be detoxification pathways and some of which may be bioactivation pathways [86]. An important example is the metabolism of the major tobacco carcinogen NNK. NNK can be metabolically activated by CYPs, but also reduced to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by CBR1 or 11-β-hydroxysteroid dehydrogenase type 1 (HSD11B1). Whereas the first phase I reaction produces the actual carcinogenic compounds, the latter facilitates the conjugation with glucuronic acid and thus excretion [89].

2.2.2 Carbonyl-metabolizing enzymes (CMEs)

The ubiquitous carbonyl moiety is often a determining factor for the biological effect of endogenous and exogenous compounds. For xenobiotic compounds, carbonyl reduction is usually regarded as a detoxification step as the resulting alcohol or hydroquinone is conjugated more easily [23, 33]. The term CMEs refers to enzymes

involved in the $NAD(P)^+$/NAD(P)H-dependent oxidation or reduction of carbonyl compounds, *i.e.* aldehydes, ketones and quinones. Human CMEs include members of several enzyme (super)families including the family of alcohol dehydrogenases (ADHs) of the medium-chain dehydrogenases/reductases (MDR)-type, the aldehyde dehydrogenase (ALDH) superfamily, the NAD(P)H:quinone acceptor oxidoreductase family (NQO) and the superfamilies of short-chain dehydrogenases/reductases (SDR) and aldo-keto reductases (AKRs) [16]. Many of those enzymes exhibit endogenous functions as well as xenobiotic activities and, in the following, an emphasis will be on such with established xenobiotic activities [23, 33, 90-93].

2.2.2.1 Overall reactions in carbonyl metabolism and cofactor specificity

With NAD(H) as main cofactor, classical ADHs and ALDHs preferably participate in oxidative pathways, while AKRs, SDR and CRYZ, mostly relying on NADP(H), tend to perform reductive routes in carbonyl metabolism (Fig. 5) [16]. This is not without exceptions to the rule. For instance, the SDR member 15-(NAD^+)-hydroxyprostaglandin dehydrogenase (HPGD) is NAD^+-specific and several AKRs with dual cofactor specificity, albeit most often still with a preference for NADP(H), have been identified [94, 95]. NQO1 is unique in the aspect that it can use both NADH and NADPH with equal efficiency for two-electron reduction of quinones (Fig. 5). In contrast, NQO2 uses dihydronicotinamide riboside (NRH) rather than NAD(P)H [96].

2.2.2.2 The multiplicity of CMEs

Members of all of the above mentioned enzyme (super-)families have multiple functions in endogenous and xenobiotic metabolism. Human ADH1, ADH2 and ADH4 enzymes contribute to various extents to the detoxification of ethanol but are also involved in the metabolism of bile acids, steroids and retinoids [91, 97-99]. ADH3 (see 2.3) is the major formaldehyde scavenger, but also plays an important role in NO homeostasis by reducing GSNO [29-32, 100]. CRYZ, to date the best-studied human member of the MDR quinone oxidoreductase (QOR) family, displays enzymatic activity towards xenobiotic *ortho*-quinones, but has also been shown to bind to AU-rich elements in RNA, implying a potential role in post-transcriptional regulation [101-104]. On a side note, the family classification of CRYZ as provided by the Swiss-Prot database (http://ca.expasy.org/sprot/), where QORs including CRYZ are classified as a subfamily under the family of zinc-containing alcohol dehydrogenases, is partially

misleading. A complete screen of six completed eukaryotic genomes for MDR identified QORs as a discrete family belonging to the non-zinc containing MDR [103].

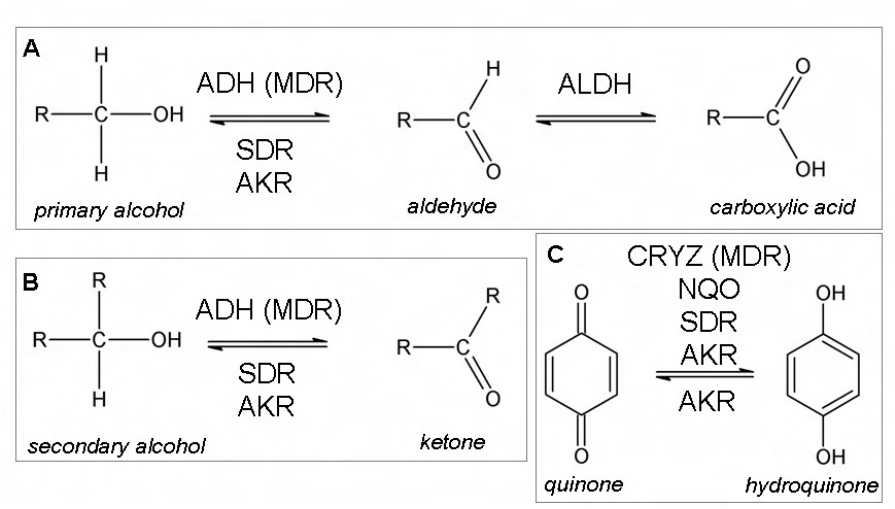


Fig. 5. Types of reactions catalyzed by families of CMEs, modified from [16]. (**A**) Primary alcohols are oxidized to aldehydes mainly by ADHs of the MDR superfamily; the reverse reaction is often catalyzed by SDR or AKRs. Aldehydes are further oxidized to carboxylic acids by ALDHs. (**B**) Secondary alcohols are oxidized to ketones mainly by the action of ADH; again, the reverse reaction is often catalyzed by enzymes of the SDR or AKR superfamilies. (**C**) ζ-Crystallin (CRYZ), belonging to the MDR family of QORs, and members of the NQO, SDR and AKR families catalyze the two-electron reduction of quinones to hydroquinones. The reverse reaction can be catalyzed by AKRs.

Endogenous substrates for ALDHs include intermediates in fatty acid metabolism (ALDH3 family, ALDH6A1), amino acid metabolism (ALDH4A1, ALDH5A1, ALDH6A1, ALDH7A1), the one-carbon pool by folate (ALDH1L1), retinoid metabolism (ALDH1, ALDH8A1), and the synthesis of the neurotransmitter γ-aminobutyric acid (ALDH9A1) [105-108]. Some ALDHs protect against aldehydes formed during lipid peroxidation, *e.g.* ALDH3A1 [109, 110]. Acetaldehyde (including after endogenous ADH-mediated oxidation of ethanol) is most efficiently oxidized by ALDH2 [105, 111]. Finally, ALDH1A1, ALDH2 and ALDH3A1 are involved in the metabolism of oxazaphosphorine anticancer chemotherapeutics as cyclophosphamide and ifosfamide [105, 112].

The SDR superfamily can functionally be subdivided into those involved in intermediary metabolism, *e.g.* UDP-galactose epimerase, dihydropteridin reductase, and sepiapterin reductase, and those involved in lipid hormone and mediator

metabolism including 3β-, 11β-, 17β-hydroxysteroid dehydrogenases, retinol-/retinal dehydrogenases and prostaglandin dehydrogenases [113]. Particularly the latter group contains many members with established additional functions in xenobiotic metabolism [23, 33, 90, 92, 114]. To name a few examples, the ubiquitous CBR1, probably the best-studied among the SDR, converts endogenous and exogenous substrates as diverse as prostaglandins, steroids, lipid aldehydes, NNK, anthracycline anticancer drugs and PAH-derived quinones [23]. Similarly, the microsomal enzyme HSD11B1 catalyzes the reversible reduction of 11-oxo glucocorticoids to 11-hydroxy glucocorticoids, but has also been demonstrated to metabolize NNK as well as cytotoxic quinones and the prospective anti-cancer drug oracin [115-117]. Finally, due to its function in the inactivation of prostaglandins, HPGD is considered to act as a tumor suppressor in many cancer types, but has also been suggested to play a role in the metabolism of PAH-derived quinones [114, 118, 119].

Although structurally unrelated, the AKRs exhibit very similar substrate specificities compared to the SDR including reduction of carbonyl functions in retinoids, steroids, eicosanoids, polyols, lipid peroxidation aldehydes and xenobiotics [23, 93]. Currently 13 human AKRs have been identified including ten cytosolic oxidoreductases, but also three potassium voltage-gated channel subunits (http://www.med.upenn.edu/akr/) [93]. Most AKRs have established functions in bioactivation/detoxification, as in the metabolism of anticancer drugs including anthracyclines (AKR1A1, AKR1C2) and oxazaphosphorines (AKR1B1) and of carcinogens including PAH metabolites (AKR1A1, AKR1C1-C4, AKR1B10), NNK (AKR1C1, C2, C4), and aflatoxin (AKR7A2, A3) [93, 120].

The NQO family (previously termed DT-diaphorases, currently also termed quinone reductases) consists of cytosolic FAD-containing enzymes that catalyze the reduction of cytotoxic quinones and nitroaromatic compounds. It comprises two human members, NQO1 and NQO2, with partly overlapping substrate specificities, but distinct cofactor requirement (as already mentioned under *2.2.2.1)* [121]. NQO1 also plays a role in maintenance of the reduced form of the endogenous antioxidant coenzyme Q [122]. NQO-mediated reactions can also lead to metabolic activation of cytotoxic agents as *e.g.* the anti-tumor prodrug CB1954 [23, 121, 123, 124]. The latter property, in combination with multiple evidence that suggests NQOs as neoplastic markers, makes them interesting chemotherapeutic drug targets [124-126]. Moreover, recent evidence

suggests that NQOs are involved in the control of proteasomal degradation of tumor suppressors including p53 [127, 128].

In summary, CMEs include several evolutionarily unrelated enzyme (super)families with central and often competing or overlapping functions in the metabolism of steroids, bile acids, prostaglandins and retinoids, as well as in the biotransformation of xenobiotic carbonyl compounds including *e.g.* ethanol, formaldehyde, NNK, PAH-derived metabolites and different groups of anti-cancer therapeutics. Hence, CMEs are implicated in carcinogenesis as well as cancer chemotherapeutic drug resistance. Interestingly, recent evidence moreover suggests non-oxidoreductase functions for some quinone reductases, *e.g.* CRYZ as regulator of RNA stability and NQOs as modulators of proteasomal tumor suppressor degradation [102, 104, 127, 128].

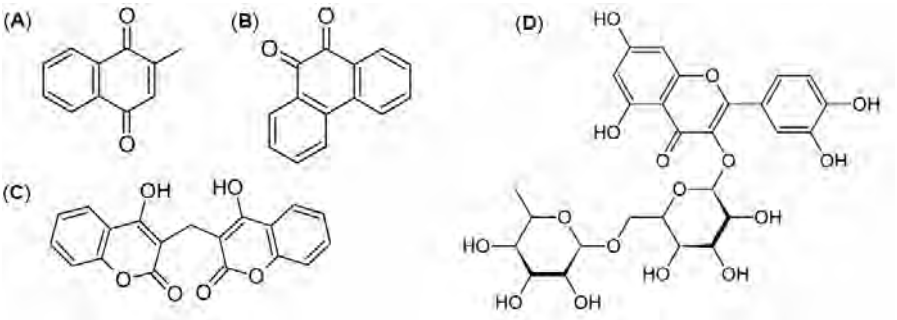


Fig. 6: Chemical structures of the substrates menadione (**A**) and 9,10-PQ (**B**) as well as the inhibitors dicoumarol (**C**) and rutin (**D**). Rutin is the glycoside between the flavonoid quercetin and the disaccharide rutinose.

As a consequence of the overlapping substrate specificities, it is a challenge to assign specific enzymes to reductive biotransformation reactions mediated by CMEs in crude protein mixtures. Under circumstances of limited material, *e.g.* when using cultured normal cells with a finite life span, a virtually collective assessment of CME-mediated xenobiotic metabolism (with the exception of ALDHs and classical ADHs) is possible by the use of two promiscuous substrate quinones, menadione and 9,10-phenanthrenequinone (9,10-PQ) in combination with NAD(P)H; notably, those two quinones are often among the best substrates for CMEs with quinone reduction activity [33, 84, 101, 104, 129-132]. Furthermore, the assessment of cofactor specificity and the use of relevant inhibitors, *e.g.* dicoumarol and rutin, can allow for some discrimination between different CME activities [18, 129]. Dicoumarol inhibits NQO1, CBRs and

CRYZ, while rutin and related flavonoids inhibit CBRs, some AKRs and NQO2 [101, 132-135]. The structures of menadione, 9,10-PQ, dicoumarol and rutin are shown above (Fig. 6).

2.2.3 Glutathione (GSH) - small but powerful

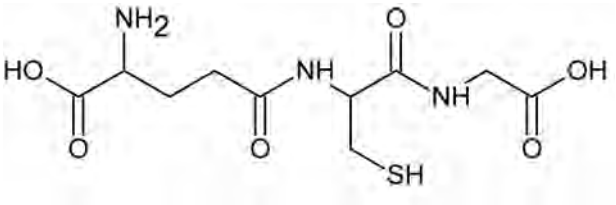


Fig. 7: Structure of glutathione (γ-glutamylcysteinylglycine)

GSH, the tripeptide γ-glutamylcysteinylglycine (Fig. 7), is found in millimolar concentrations in most cells and is one of the most important low molecular weight compounds in the defense against reactive metabolites and xenobiotics [86, 136]. The reactive cysteine thiol group in GSH forms the basis of its cytoprotective function. Scavenging of radicals and reactive metabolites by GSH often occurs spontaneously by donation of the thiol hydrogen or proton, respectively, most commonly followed by the reaction with another GSH molecule to form glutathione disulfide (GSSG). If a peroxide is the reactive metabolite, this reaction is catalyzed by the enzyme glutathione peroxidase. Under normal redox conditions, GSSG is recycled to GSH by GSSG reductase under concomitant oxidation of NADPH. Under conditions of oxidative stress, however, reactive oxygen species accumulate and GSH and NADPH are depleted, making the cell more vulnerable to toxic challenge. GSH depletion contributes to the pathogenesis of a number of disease conditions including cancer, neurodegenerative and cardiovascular diseases [137, 138].

2.2.4 Glutathione transferases (GSTs)

In some cases, the formation of GSH conjugates can occur spontaneously, *e.g.* the GSH adduct of formaldehyde, S-hydroxymethylglutathione (HMGSH, an important intermediate in formaldehyde detoxification), is formed non-enzymatically [86]. Yet for the most part, GSTs catalyze the nucleophilic attack of GSH on electrophilic atoms of hydrophobic xenobiotic compounds and lipid peroxidation products and thus contribute to the detoxification of numerous carcinogenic, mutagenic and toxic compounds [139]. There are two major types of mammalian GSTs, the soluble GSTs which belong to the multi-gene GST superfamily and the microsomal GSTs which

belong to the MAPEG (Membrane-Associated Proteins in Eicosanoid and Glutathione metabolism) family [140]. Their catalytic activity relies on the ability to bring GSH in proximity and correct orientation in respect to the hydrophobic substrate as well as to lower the pK_a of the GSH thiol group when it is bound to the active site (from ≈ 9 in solution to ≈ 6-7 upon binding). Hence, at physiological pH, GST-bound GSH tends to occur in the more reactive thiolate anion form (GS^-) [141, 142]. Glutathionesulfonic acid, occurring as the sulfonate at physiological pH in solution, is a potent and in respect to GSH competitive inhibitor of GSTs [143, 144]. GSNO appears to be able to modulate GST activity by two mechanisms, competitive inhibition and covalent modification, presumably by S-nitrosation of a cysteine side chain [145].

2.2.5 Expression of xenobiotic enzymes in buccal keratinocytes

Previous studies have assessed expression of CYPs, GSTs and other phase II biotransformation enzymes in NBK, SVpgC2a and SqCC/Y1. Overall, these studies suggested that NBK, similar to normal tissue specimens, expressed CYP1A1, CYP1A2, CYP2C, CYP2D6, CYP2E1 and CYP3A, albeit for the most part at levels close to detection limit [146, 147]. Phase II enzymes were more abundantly expressed, particularly sulfotransferase 1A3, microsomal GST and GST P1. CYP3A was lost in the immortalized buccal cell line SVpgC2a and CYP2C, CYP2D6 and CYP3A4/7 were lost in SqCC/Y1. In contrast, CYP1A1 and CYP1B1 were elevated in SVpgC2a [147].

Furthermore, early generation microarray analysis was applied to investigate CME transcription in cultured normal and transformed buccal keratinocytes [148]. This study indicated expression of ADHs, ALDHs, SDR and AKRs including such with established functions in xenobiotic metabolism, *e.g.* ADH3, CRYZ, ALDH2, CBR1 and AKR1A1. Expression of several of the detected CME transcripts was altered in the transformed cell lines. Expression of ADH3 was assessed on transcript, protein and activity level in oral tissue, NBK, SVpgC2a and SqCC/Y1 including in organotypic culture [27, 149]. These studies revealed that ADH3 mRNA is confined to the basal and parabasal layers, while the ADH3 protein was detected at similar levels throughout the epithelia, including those generated *in vitro*. This suggested that ADH3 transcription might be regulated in association with proliferation or TSD while the protein is sufficiently stable to last throughout tissue turnover.

2.3 ALCOHOL DEHYDROGENASE 3

The highly conserved and ubiquitous ADH3 enzyme is considered to represent the ancestral form of the MDR-type ADHs [150-155]. Similar to many other CMEs, ADH3 exhibits activity towards endogenous as well as exogenous compounds, of which the most important appear to be HMGSH and GSNO. By catalyzing the oxidation of HMGSH, the spontaneous GSH conjugate of formaldehyde, ADH3 is central to the detoxification of endogenous and exogenous formaldehyde, including in the buccal mucosa [27, 91, 100]. In addition, during the last decade evidence has accumulated that strongly suggests that ADH3, through the irreversible GSNO reductase activity, affects the transnitrosation equilibrium between GSNO and S-nitrosated proteins and thus plays an important role in endogenous NO homeostasis [29, 30, 32]. Other functions attributed to ADH3 include first-pass ethanol metabolism, contribution to retinoic acid formation and oxidation of ω-hydroxy fatty acids [26, 156-158].

2.3.1 ADH3 - a protein of many names

Nomenclature of the ADHs, a family comprised of several classes including different isozymes, is still a subject under debate (http://ca.expasy.org/sprot/) [159, 160]. In line with ADH family classification, ADH3 is frequently termed "class III alcohol dehydrogenase" in the literature. As most mammalian ADHs are dimeric molecules, they have also often been named according to their individual subunits, facilitating the distinction between homo- and heterodimeric enzymes. In this context, ADH3 is referred to as "χχ ADH". Based on its catalytic activities, ADH3 has been called "glutathione-dependent formaldehyde dehydrogenase" and "GSNO reductase" (GSNOR) [32, 161]. Finally, further complexity is added by the fact that the gene nomenclature was not updated in parallel with the protein nomenclature and the gene coding for human ADH3 is currently still named *ADH5* according to the Swiss-Prot database (http://ca.expasy.org/sprot/).

2.3.2 Structure/function relationships

ADH3 exhibits a homodimeric structure with two zinc atoms per 40 kDa subunit, each comprised of two domains, designated the catalytic and the coenzyme binding domain (Fig. 8). While one of the zinc atoms is considered to serve a structural function only, the other zinc atom functions as a Lewis acid and activates the substrate in the

active site which is located in a cleft between the catalytic domain and the coenzyme binding domain [162]. These overall structural properties are shared by all classical mammalian ADH structures. However, relative to other ADHs, the structure of ADH3 shows distinct differences in the immediate vicinity of and within the active site and moreover displays a semi-open domain conformation, features that are reflected in substrate specificity and kinetic properties. More precisely, residues 55-61 and 112-120 adopt a different secondary structure and move away from the catalytic cleft, which leads to an enlargement of the active site and generates a broader entrance to the substrate-binding pocket than in the ADH1 enzymes [162]. Hence the active site cannot be saturated with ethanol, but accommodates larger substrates such as HMGSH, GSNO, medium-chain alcohols, aldehydes and ω-hydroxy fatty acids [26, 91, 150, 163]. Furthermore, several ADH3-specific residues within the active site play critical roles in ligand binding. The efficient binding of HMGSH (see Fig. 9 for the chemical structure) is mainly mediated by R114, interacting with the glycyl branch carboxyl group, the residues D55 and E57, interacting with the amino group of the γ-glutamyl branch, as well as the active site zinc interacting with the hydroxyl group (Fig. 8) [162, 164, 165].

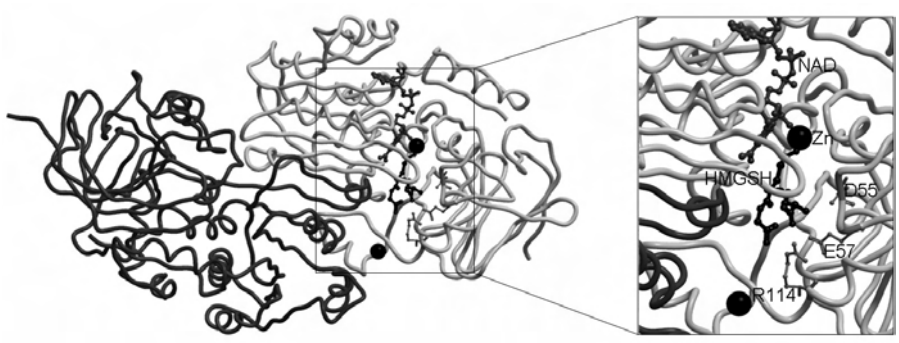


Fig. 8: Left: The dimeric structure of ADH3, showing the different monomers (dark-gray and light-gray) and the position of zinc atoms (black), NAD (dark-gray) and HMGSH (black) in the light-gray monomer. Right: Close-up view of the active site with NAD and HMGSH, demonstrating orientation of the substrate between the active-site zinc and the anion-binding pocket, represented by R114. Amino acids interacting electrostatically with HMGSH, i.e. D55, E57 and R114, as well as both zinc atoms are displayed. Only the active site zinc ion is labeled Zn. The figure was generated from pdb-file 1MC5 using Molsoft ICM-Browser [165].

Tertiary structure differences between the apoenzyme, binary and ternary complexes are consistent with a random bi-bi kinetic mechanism observed for oxidation of HMGSH and 12-hydroxydodecanoic acid under concomitant conversion of NAD^+ [163, 165-167]. Binding of the substrate or NAD(H) does not entail significant domain movement as observed upon coenzyme binding in other ADHs where an ordered bi-bi kinetic mechanism is predominant. In contrast, domain closure is executed after ternary complex formation which allows random addition of substrate and coenzyme in the course of catalysis [167].

2.3.3 Formaldehyde detoxification

Formaldehyde, the simplest carbonyl compound, has recently been classified as a human carcinogen [24]. Owing to the polarized carbonyl group, formaldehyde can be attacked by nucleophiles and electrophiles and, hence, participate in various substitution and addition reactions. A cellular environment holds various potential reactants for formaldehyde: For instance, thiols readily form hemithioacetals with formaldehyde as *e.g.* in the reaction with GSH yielding HMGSH, which can be regarded as a first-line defense against formaldehyde toxicity. In contrast, the reaction with amines results in Schiff bases which initiates the formation of DNA-DNA, DNA-protein and protein-protein crosslinks where amino groups present in DNA and proteins are stably connected via methylene bridges derived from formaldehyde [168]. The high DNA reactivity manifests itself in mutagenic effects and chromosomal changes which, in combination with formaldehyde-associated activation of proliferation, are considered to underlie formaldehyde carcinogenicity [24, 169]. Moreover, formaldehyde is a potent respiratory irritant with well-established bronchoconstricting and asthma-exacerbating effects; the underlying molecular causes, however, are poorly understood [170-173].

Formaldehyde is ubiquitous in our environment, as for instance released from plywood, present in tobacco smoke or in car exhaust. Moreover, certain occupational groups including workers in wood industry, garment industry, pathologists and embalmers can be particularly exposed to formaldehyde [24, 170]. Formaldehyde can also be formed endogenously as a result of normal metabolic pathways (*e.g.* in serine and glycine metabolism) or in the course of biotransformation processes, as for

example by oxidative demethylation of xenobiotics by CYPs or oxidation of ingested methanol [174-178].

In humans, formaldehyde can be directly metabolized by some ALDHs, but these enzymes exhibit Michaelis constants for free formaldehyde that are by several magnitudes higher than the one ADH3 exhibits for HMGSH [179-181]. Under typical cytosolic redox conditions, *i.e.* with GSH present in millimolar concentrations and a high NAD^+/NADH ratio, ADH3 is certainly the most efficient formaldehyde-metabolizing enzyme and thus, together with esterase D (ESTD, also termed S-formylglutathione hydrolase) constitutes the primary defense against formaldehyde damage (Fig. 9) [182]. Several functional studies uniformly support the importance of ADH3 for formaldehyde resistance in prokaryotic as well as in eukaryotic organisms: Formaldehyde-resistance in an *E. coli* strain is mediated by a plasmid carrying the gene for an ADH3-homologue [183, 184]. Overexpression of *Arabidopsis thaliana* ADH3 in yeast and plants confers improved resistance towards exogenously added formaldehyde [185]. Finally, the LD_{50} for formaldehyde in Adh3-deficient mice is significantly lower than the one in wild-type mice [100].

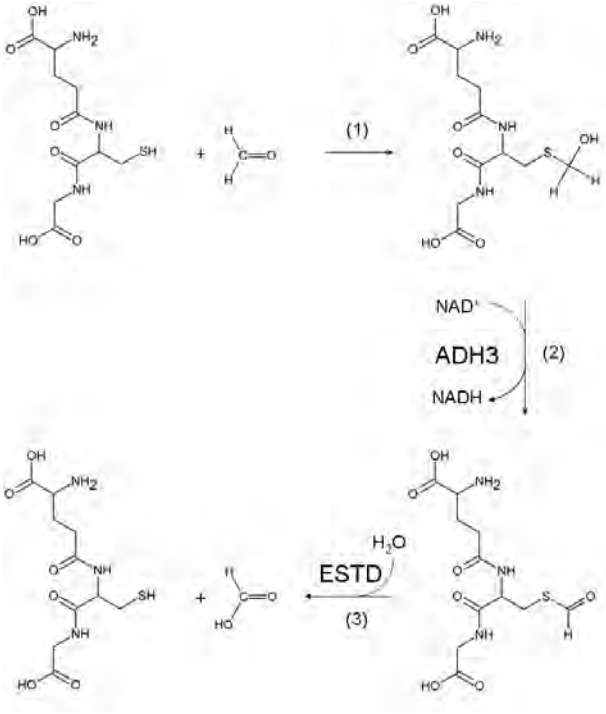


Fig. 9: ADH3-mediated formaldehyde detoxification pathway. (1) Formaldehyde is non-enzymatically conjugated with GSH to yield HMGSH, which (2) is oxidized by ADH3 under concomitant conversion of NAD^+ to give S-formyl-glutathione. This step is followed by (3) ESTD-mediated hydrolysis which regenerates GSH and yields formate.

2.3.4 Reduction of S-nitrosoglutathione

2.3.4.1 Role in nitric oxide (NO) signaling

NO signaling is involved in a large array of physiological functions including vasodilation, cell death, regulation of redox potential and control of respiration [186]. NO is formed endogenously by the action of several NO synthases which convert the amino acid arginine to citrulline and NO (Fig. 10). With a biological half-life of only a few seconds, NO is relatively short-lived and NO bioactivity is to a large part conveyed by post-translational modification of proteins, by metal nitrosation or S-nitrosation [186, 187]. Hence, intracellular NO signaling is in part mediated through protein S-nitrosation with currently more than 100 identified potential targets, including proteins involved in cellular processes as diverse as apoptosis, membrane trafficking and iron homeostasis [186].

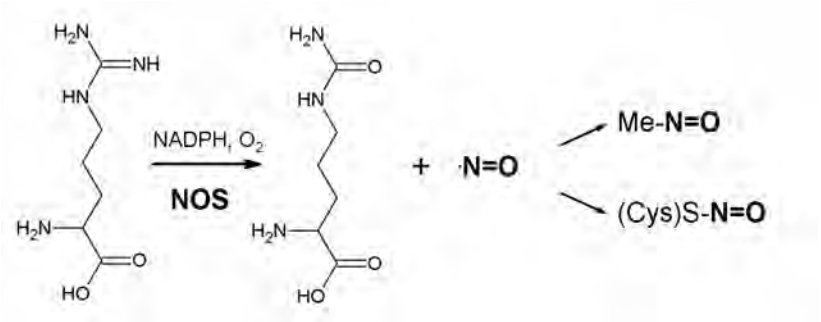


Fig. 10: Nitric oxide synthases convert arginine to citrulline and NO under concomitant consumption of NADPH and O_2. Then, NO can modify proteins by covalently binding to metal centers (metal nitrosation) or cysteine residues (S-nitrosation).

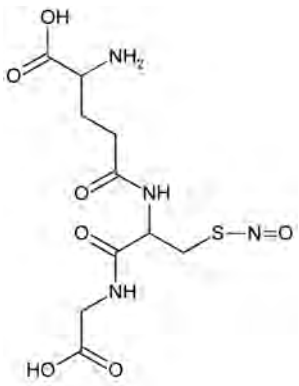


Fig. 11: Structure of GSNO.

S-nitrosated proteins appear to exist in transnitrosation equilibrium with GSNO, the most common low molecular weight S-nitrosothiol (SNO) (Fig. 11) [188]. During the last years, evidence has emerged that supports the concept that this equilibrium is affected by ADH3 through its GSNO reductase activity (Fig. 12) [30, 32]. Notably, the ADH3-mediated GSNO reductase activity is the only GSNO-converting activity detected so far that does not result in NO release, in contrast to glutathione peroxidase, the thioredoxin system, xanthine oxidase and Cu/Zn superoxide dismutase [28, 29, 189-192]. Instead, the NO moiety is reduced and irreversibly removed from the NO pool, compatible with an NO terminase function [28, 29]. Furthermore, it is by magnitudes the most efficient GSNO-converting activity among all suggested activities, provided the appropriate cofactor NADH [28, 29, 189-192]. Also functional studies support this concept: In cultured cells, the degree of downregulation of ADH3 by RNA interference correlated inversely with SNO levels [30]. Moreover, Adh3-deficient mice show significantly increased SNO levels in lung homogenates [32]. Thus, in its function as GSNO reductase, ADH3 appears to indirectly govern levels of protein SNOs and is therefore likely to serve an important regulatory function in NO signaling.

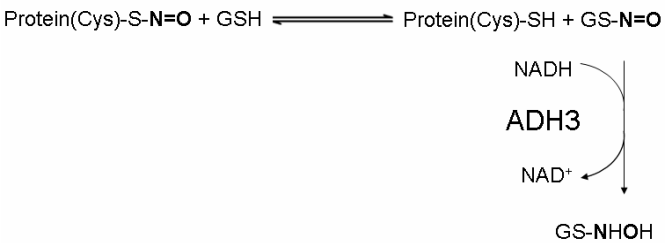


Fig. 12: The transnitrosation equilibrium between protein SNOs and GSNO is shifted towards the right-hand side by ADH3-mediated GSNO reduction under concomitant conversion of NADH.

Deregulated levels of cellular SNOs are often associated with medical conditions including cerebral ischemia, Alzheimer's disease, sclerosis, and asthma [32, 193-196]. Notably, genetic and functional evidence support the involvement of ADH3 in asthma. GSNO functions as an endogenous bronchodilator in airway lining fluid and performs as such a protective role in asthma [197]. In wild-type mice exhibiting airway hyperresponsivity, allergen challenge results in increased GSNO reductase activity in airway lining fluid and SNO depletion in adjacent lung cells. In contrast, Adh3-deficient mice are protected from airway hyperresponsivity [32]. In addition, two single

nucleotide polymorphisms in the gene coding for ADH3 have been coupled to asthma susceptibility [161].

2.3.4.2 Product formation following GSNO reduction

ADH3-mediated GSNO reduction results in a transient semimercaptal, S-hydroxylaminoglutathione (cf. Fig. 12), but conclusions from the literature have diverged about the next step. The spontaneous rearrangement to glutathione sulfinamide or the reaction with GSH to form GSSG as the final product have been suggested [28, 29, 31]. In addition, different reaction mechanisms with GSH have been put forward. One study provided evidence for equimolar consumption of NADH and GSNO as well as the direct interception of the intermediate by GSH to yield GSSG and hydroxylamine [28]. In contrast, another study suggested the reduction of S-hydroxylaminoglutathione to S-aminoglutathione under concomitant oxidation of a second NADH molecule, followed by the reaction with GSH to yield GSSG and ammonia [31].

3 AIMS OF THIS THESIS

The aims of this thesis were two-fold. The first overall aim was to contribute to the elucidation of buccal carcinogenesis mechanisms and to discover novel multiple tumor markers and drug targets with the long-term goal to propose novel diagnostic and therapeutic strategies for BSCC. These questions were addressed in the described *in vitro* model for buccal carcinogenesis including proliferative and differentiated states. Here, the specific aims were:

- to assess overall differential gene expression in the two transformed buccal keratinocyte lines SVpgC2a and SqCC/Y1 relative to NBK
- to test a combination of bioinformatics tools for the integration of gene expression data including the detection of tumor suppressor/promoter genes as well as novel multiple biomarkers including molecular networks and gene ontology (GO) categories
- to establish protocols for the selective culture of proliferative and variably differentiated NBK in order to mimic the process of buccal TSD in culture and assess whether *ADH3* transcription associates with proliferation or TSD
- to analyze expression and activity of CMEs in SVpgC2a and SqCC/Y1 relative to NBK as well as in association to the process of buccal TSD

The second overall aim was to assess the major buccal formaldehyde scavenger ADH3 in terms of exogenous/endogenous substrate competition, *i.e.* in its dual function as formaldehyde dehydrogenase and GSNO reductase. Here, the specific aims were:

- to assess how formaldehyde affects ADH3-mediated GSNO reduction
- to study product formation following GSNO reduction as a function of GSH concentrations
- to test the products of ADH3-mediated GSNO reduction as GST inhibitors
- to examine inhibition of ADH3-mediated GSNO reduction by different substrate analogues

4 EXPERIMENTAL PROCEDURES

The following constitutes a short summary of the various methods used in the present thesis work. For more detailed descriptions, I would like to refer to the attached original papers I-V and references therein.

Cell culture of NBK, SVpgC2a and SqCC/Y1 was in a serum-free medium with high levels of amino acids (EMHA, Papers I-IV). Post-confluent culture in absence or presence of FBS was used for induction of TSD (Papers II and III). The colony-forming efficiency (CFE) assay was used to assess proliferative ability of NBK (Paper II). Formaldehyde exposure was in a chemically defined variant of EMHA and followed by the determination of intracellular SNOs by a photometric assay involving Cu^{2+}-triggered release of NO and subsequent diazotization and derivatization with sulfanilamid and *N*-(1-naphthyl)ethylenediamine (Saville-Griess assay, Paper IV).

Assessment of gene expression at transcript level was performed by DNA microarray analysis (Papers I and III) or standardized reverse transcriptase-polymerase chain reaction (StaRT-PCR, Paper II). Assessment at protein level was performed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), subsequent in-gel digestion and matrix-assisted laser/desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) followed by mass fingerprinting (Paper I). For some low-abundant proteins also Western Blot analysis was performed.

Bioinformatics processing of large-scale gene expression data included the sequential use of three bioinformatics tools, the Gene Ontology Tree Machine (GOTM), AffyAnnotator, and Ingenuity Pathway Analysis (IPA, Paper I). The GO is a controlled vocabulary, developed and maintained by the Gene Ontology Consortium (http://www.geneontology.org/) that sorts gene products into categories according to their associated biological processes, molecular functions and cellular components. The web-based tool GOTM uses a statistical hypergeometric test to analyze for enrichment of such categories in any interesting data set (http://bioinfo.vanderbilt.edu/gotm/). AffyAnnotator, developed at the Bioinformatics and Expression Analysis Core facility at Karolinska Institutet, allows for visualization and sorting of transcripts on Affymetrix chips according to the GO (http://www.bea.ki.se/jnlp/). IPA can be applied

to search for molecular networks and pathways that are common to any list of interesting genes. IPA relies on a large knowledge database consisting of information about interactions between gene products which has been extracted from the literature (IPA 4.0, Ingenuity Systems, http://www.ingenuity.com).

Synthesis of substrates and inhibitors: GSNO was synthesized according to Hart [198] (Papers IV and V). Glutathione sulfinamide and sulfinic acid were obtained following ADH3-mediated reduction of GSNO and separation of the products by anion exchange chromatography. Product fractions were identified by electrospray ionization mass spectrometry (ESI MS, Paper IV). S-acetamidoglutathione was synthesized by acetamidylation of GSH with iodoacetamide, resolved on an anion exchange column, and its identity was verified by ESI MS (Paper V). Concentrations of glutathione sulfinamide, sulfinic acid and S-acetamidoglutathione were determined by ninhydrin-based amino acid analysis (Papers IV and V).

Enzymatic activity for dehydrogenase/reductase reactions was mostly assessed spectrophotometrically by following the concomitant conversion of NAD(P)(H) and/or GSNO at 340 nm (Papers III-V). In paper IV, also fluorescence spectrophotometry (λ_{exc} = 340 nm, λ_{em} = 455 nm) was used for this purpose. Microsomal glutathione transferase 1 (MGST1) activity was monitored by following the formation of the GSH-dinitrobenzene conjugate from GSH and 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm. **Protein-ligand docking simulations** were performed with ICM 3.5 (Molsoft LLC) (Paper V) [199-201].

Quantitative **product analysis** of GSNO reduction was performed by resolving the reaction mixtures on a strong anion exchange column followed by peak integration. Peak intensities were calibrated for commercially available reactants and products. Identities of reactant/product fractions were verified by ESI MS. Hydroxylamine concentrations were determined with a modified form of a colorimetric assay where hydroxylamine is trapped as a quinoline oxime [202].

5 RESULTS AND DISCUSSION

5.1 DIFFERENTIAL GENE EXPRESSION IN TRANSFORMED BUCCAL KERATINOCYTES RELATIVE TO NBK

5.1.1 Differentially expressed proteins are confirmative of the model system and include potential novel tumor markers (Paper I)

Protein profiling by 2D-PAGE, subsequent in gel digestion and MALDI-TOF MS for mass fingerprinting led to the identification of 19 differentially expressed proteins including several isoelectric variants in one or both of the transformed cell lines SVpgC2a and SqCC/Y1. In agreement with decrease or loss of CKs as well-established characteristics of oral SCC, CK5, CK14, CK17 and CK6 were essentially lost in SVpgC2a and CK7 was significantly decreased in SVpgC2a and SqCC/Y1 [39, 203, 204]. In contrast, the heat shock proteins (HSPs) HSP27, HSP60 and HSP70 were upregulated in one or both of the transformed cell lines. All three HSPs have been found overexpressed in oral premalignant lesions and carcinomas and have thus previously been considered as diagnostic markers [61, 205-207]. Notably, novel aspects in that context were the observed selective down- and upregulation of distinct isoelectric variants of CK5 and HSP60, respectively, in the BSCC cell line. Additional proteins implicated from this study previously considered as buccal or oral tumor markers included heterogeneous ribonucleoprotein (hnRNP) B1, stratifin (SFN) and α-enolase [208-210]. In oral SCC, SFN, an epithelium-specific p53-transactivated tumor suppressor gene, can be inactivated by either loss of p53 function or promoter methylation and loss of SFN expression appears to occur early in carcinogenesis [209, 211]. In contrast, a study on differential protein expression in BSCC tissue specimens demonstrated strong upregulation of SFN, which, however, might reflect the specified tissue heterogeneity rather than tumor-associated changes [210]. Notably, the remaining altered proteins, namely acetyl-CoA acetyltransferase (ACAT1), elongation factor-Tu, ferritin light chain, fumarate hydratase, glutamate dehydrogenase, hnRNP C1/C2, peroxiredoxin 3 and tropomyosin 3 had not been implicated in previous studies on buccal or oral SCC and thus constituted potential novel markers of BSCC.

5.1.2 Bioinformatics processing identifies further multiple and integrative biomarkers for BSCC (Paper I)

Of the discussed differentially expressed proteins identified by 2D PAGE expression only some were already altered at the transcript level including all CKs, SFN and ACAT1. Otherwise, changes in protein and transcript levels for individual genes were mostly inconsistent. For HSP27 and HSP70 changes in transcript and protein levels even anti-correlated, possibly reflecting autoregulation of HSP transcription by abundant HSPs [212]. Generally, transcript assessment is considered to capture only about 40 % of the changes in protein expression [213]. Hence, the results confirm that transcript data should be interpreted with caution in tumor marker studies when focused on single gene markers. However, differential gene expression on mRNA level often reflects protein abundance better in functional categories than on single gene level [214]. Accordingly, functional classification by bioinformatics tools can allow for bridging the gap between transcript and protein data.

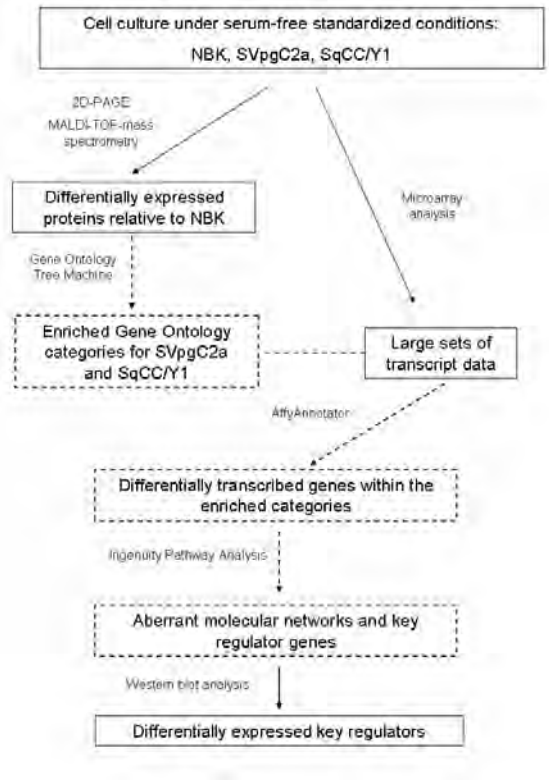


Fig. 13: Scheme of overall experimental approach and data interpretation. NBK, SVpgC2a and SqCC/Y1 were cultured under a standardized serum-free condition and differential expression was assessed by 2D-PAGE and DNA microarray analysis. A sequence of three bioinformatics tools (GOTM, AffyAnnotator and IPA, indicated by dashed lines) was used to analyze the data sets. Finally, expression of some resulting key regulators was assessed by Western blot analysis.

Here, a strategy using three bioinformatics tools was devised (Fig. 13): The differential protein signatures of each cell line relative to NBK were used for GOTM-based enrichment for functional and structural categories which yielded eight and four GO categories for SVpgC2a and SqCC/Y1, respectively (shown for SqCC/Y1 in Fig. 14). Target genes within the resultant GO categories were then selected from the transcript data by AffyAnnotator. A representative AffyAnnotator-derived heat map shows the results for transcripts within the GO category response to protein stimulus in SqCC/Y1, including the six transcripts thereof selected for further analysis (Fig. 15). Finally, within the pooled transcript data from all enriched GO categories for each cell line, IPA identified aberrant molecular networks and their key regulator genes. The altered transcripts in SVpgC2a and SqCC/Y1 generated 10 and 6 highly significant molecular networks, respectively (see Fig. 16 for an example). For a majority of the networks in both cell lines, IPA suggested "cancer", "cell death" or "cellular growth and proliferation" as one of the associated top three functions.

Next, screening of these molecular networks for key regulator genes exhibiting at least three interactions with altered transcripts resulted in the identification of 18 potential tumor suppressor/promoter genes. Most hereby identified transcription factors are known to exhibit multiple roles in cancer causation. The key regulator genes implicated with both lines, *CDKN2A*, *TP53*, *MYC*, *MYCN* and *SP1* represented genes and markers for involvement in various oral cancers, including BSCC [4, 61, 215-218]. Eight further genes identified from one of the transformed cell lines, *TP73L*, *SMAD4*, *RELA*, *PPARG*, *ARNT2*, *EPAS1*, *HIF1A* and *SP3* have previously been shown to directly or indirectly associate to oral or buccal carcinoma [4, 61, 62, 217-222]. In contrast, deregulation of *CEBPA*, *HNF4A*, *SIM1*, *NFYB* and *MYOD1* or related proteins/functions was not previously implicated for buccal/oral cancer and thus, those genes constitute potential novel key regulator genes for buccal carcinogenesis.

Five of these 18 transcription factors indicated as deregulated through IPA were subjected to Western blot analysis. Relative to NBK, expression of transcription factors Sp1 (*SP1*), Sp3 (*SP3*) and c-Myc proto-oncogene (*MYC*) was increased in both SVpgC2a and SqCC/Y1. Differently, hypoxia-inducible factor 1α (*HIF1A*), implied from IPA as deregulated in SVpgC2a, was increased selectively in SqCC/Y1 and p16 (*CDKN2A*), implied from IPA for both transformed cell lines, was increased selectively in SVpgC2a. Although in part seemingly contradictory, this might actually reflect the

fact that aberrant key regulator function only variably is a result of altered transcript or protein levels, but also involves other mechanisms as *e.g.* post-translational modifications or inhibition of nuclear translocation [223]. Notably, changes in transcript and protein levels were mostly inconsistent also for these five key regulator genes; qualitative agreement was only observed for transcript and protein levels of p16.

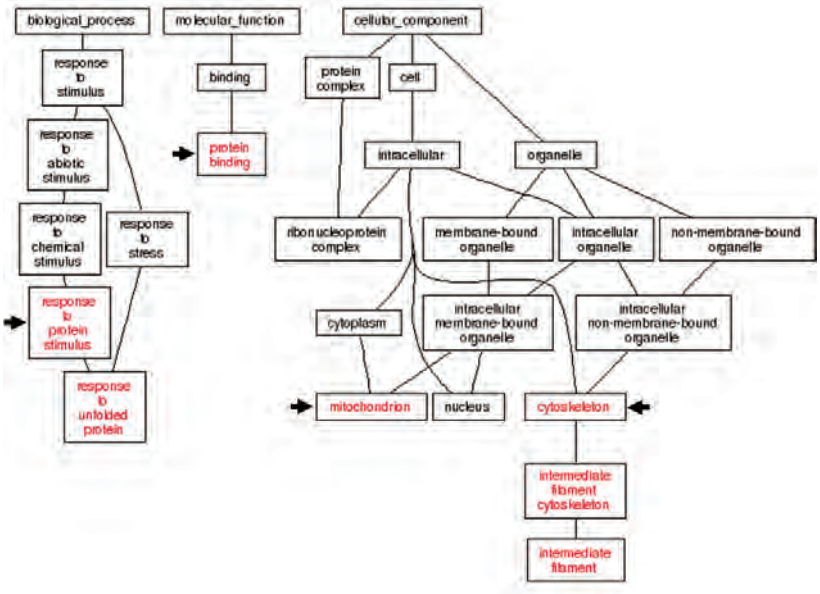
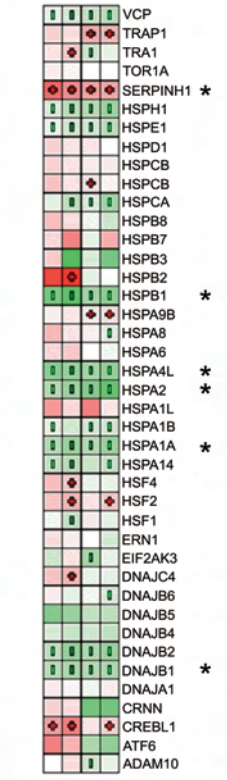
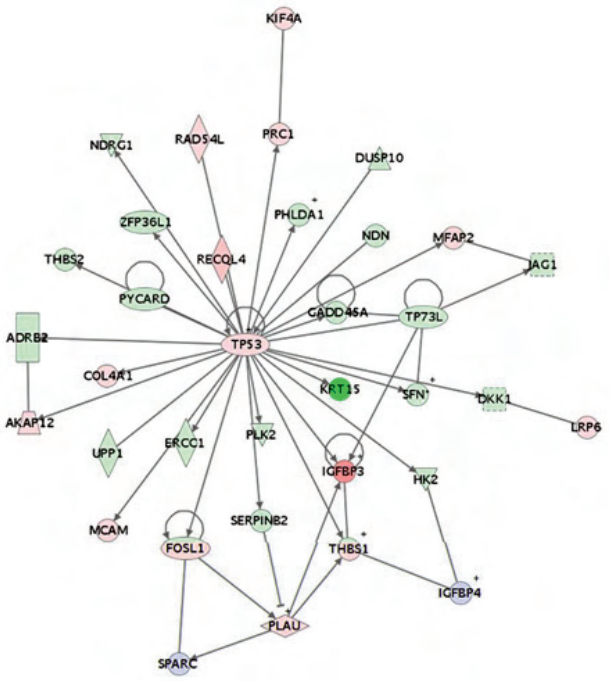


Fig. 14: GOTM data output exemplified for SqCC/Y1 relative to NBK. A total of seven enriched GO categories were identified (displayed in red) and the four at the highest hierarchical level (depicted with arrows) were used in further analysis.

In summary, the described approach led to the identification of multiple potential markers for BSCC, both at transcript and at protein level, as well as of several potential integrative biomarkers such as GO categories and deregulated molecular networks including their aberrant key regulator genes. Expression changes in a relatively low number of abundant proteins, combined with the use of bioinformatics tools and the inclusion of large-scale transcript data for which little material is required, ultimately allowed for identification of low-abundance transcription factors. Hence, the methodology seems useful for the analysis of gene expression data under circumstances of limited samples, *e.g.* in personalized medicine.



◀Fig. 15: AffyAnnotator-derived heat map for the GO category response to protein stimulus, enriched in SqCC/Y1 relative to NBK (see Fig. 14). Four pair-wise comparisons are shown. Significant Fold Changes are visualized with plus or minus signs and transcripts significantly deregulated in all four comparisons (marked with an asterisk) were chosen for further analysis. Red or green color, respectively, indicates increased or decreased transcripts.



◀Fig. 16: Example of a molecular network generated by IPA from altered transcripts within enriched GO categories for SVpgC2a relative to NBK. Here, *TP53* and *TP73L* are central key regulators. Red or green color, respectively, indicates increased or decreased transcripts.

5.1.3 SVpgC2a and SqCC/Y1 exhibit altered quinone reduction potential (Paper III)

CME gene transcription was assessed in the transformed cell lines relative to NBK and a total of 8 CME genes were shown to be differentially transcribed in SVpgC2a and/or SqCC/Y1. Transcripts of *CRYZ*, *ALDH1A3*, *ALDH2*, *ALDH4A1*, *GALE*, *HSD17B2* and *QDPR* were decreased in one or both of the transformed cell lines and transcripts of *ALDH3B1* were increased in both. These results were in very good agreement with an earlier generation microarray analysis [148]. CME-mediated quinone reduction activity in crude cell lysates of NBK, SVpgC2a and SqCC/Y1 had not been studied previously.

As to SVpgC2a, overall menadione reduction activity was unchanged relative to NBK, but not inhibited by dicoumarol, excluding CBR1 or NQO1 as significant contributors to that activity [132, 134]. At the same time, rutin-inhibitable 9,10-PQ reduction was upregulated, which suggests increased AKR activity [135]. Thus, the immortalized cell line SVpgC2a exhibited altered competence for quinone reduction relative to NBK, both in terms of specificity and potency. Differently, in SqCC/Y1 relative to NBK, NQO1-like (*i.e.* dicoumarol-sensitive and characterized by dual cofactor specificity) was upregulated and overall 9,10-PQ reduction activity increased, but, in contrast to NBK, with unaltered rutin-inhibitable fraction. The underlying experimental design does not allow for specific enzyme assignments of any of these activities, but nevertheless the results imply that quinone reduction competence is altered in association with cell transformation. The possible implications for buccal drug response and for the use of continuous cell lines in toxicity testing emphasize the need for further studies.

5.2 DIFFERENTIAL GENE EXPRESSION IN PROLIFERATIVE AND DIFFERENTIATED BUCCAL KERATINOCYTES

5.2.1 Confluency with or without FBS induces TSD in buccal keratinocytes (Papers II and III)

In paper II, different culture protocols were used to induce TSD in NBK. The course of this process was monitored by assessment of morphological changes under the microscope, assessment of proliferative ability by use of the CFE assay, and finally by

assessment of established markers of proliferation and TSD. Maintaining NBK at confluency for five days induced a squamous morphology, inhibited CFE, decreased transcription of two proliferation markers, cell division control protein 2 (CDC2) and proliferating cell nuclear antigen (PCNA), and increased transcription of two markers of TSD, involucrin (IVL) and small proline-rich protein (SPR) 1. Addition of 2 % and 10 % FBS variably influenced these effects: First, inhibition of CFE and loss of CDC2 and PCNA transcription was partly counteracted. Second, IVL, but not SPR1 transcription, was promoted. Third, transcription of CK5, a marker for basal keratinocytes, was only decreased in FBS-containing post-confluent cultures. After that, continued culture for ten and 15 consecutive days gradually abolished CFE in absence and presence of FBS, but only few additional transcriptional changes were observed, including the loss of CK5 also in post-confluent cultures without FBS and a strong increase in IVL transcription in the presence of 2 % FBS.

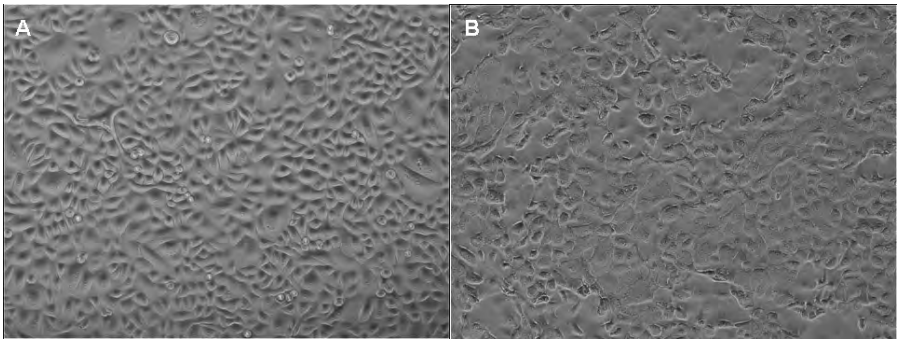


Fig. 17: Morphologies of NBK grown to a confluent state in serum-free medium (**A**) and maintained at confluency for three days with 5 % FBS (**B**).

Paper III aimed at assessing early changes in TSD. Thus, a culture protocol including 5 % FBS was applied in order to enrich for a proliferative keratinocyte phenotype at the onset of TSD. Cells were collected when they exhibited the typical flattened squamous morphology associated with the onset of TSD which usually was after three days post-confluent culture (Fig. 17). Markers of proliferation were largely unaffected under this growth protocol, while markers of early TSD (IVL and SPR3, gene name *SPRR3*) and one marker of suprabasal keratinocytes (CK13, gene name *KRT13*) were drastically increased (Fig. 18). Expression of a marker of late TSD (TGM1) and another marker of suprabasal keratinocytes (CK4, gene name *KRT4*) was less promoted, with a trend to increased levels that failed significance (Fig. 18). Here,

in order to distinguish between TSD-associated and serum-related effects, SVpgC2a and SqCC/Y1, both TSD-deficient, were subjected to the same growth protocol. As expected, those cell lines failed to regulate all markers of TSD in response to serum (Fig. 18). The overall results demonstrate that different culture protocols can be used in efforts to selectively enrich proliferative and differentiated keratinocyte phenotypes, *i.e.* different *strata* in a normal buccal mucosa.

5.2.2 *ADH3* transcription associates with proliferation (Paper II)

Several growth protocols involving post-confluent culture for up to 15 days with and without FBS were used to study the association of *ADH3* transcription with markers of proliferation and/or TSD. Transcript levels were assessed by StaRT-PCR technique. Loss of transcripts for ADH3 in post-confluent cultures showed good positive correlation with loss of proliferation markers, while neither a positive correlation with CK5 nor a negative correlation with the TSD markers was evident. Hence, *ADH3* transcription associated with proliferation and could thus serve as a proliferation marker.

In this context it should be emphasized that only ADH3 mRNA levels associate with proliferation. In contrast, it has been demonstrated that the ADH3 protein is not confined to (para-)basal layers, but maintained in active form throughout the entire keratinocyte life span [27]. This might simply constitute an energy-saving mechanism where unnecessary mRNA synthesis is avoided once the protein is abundant and stable. Additionally, preserving actively formaldehyde-scavenging ADH3 throughout the epithelium, while confining transcription to the basal tissue compartment, the least affected by formaldehyde and other genotoxic agents, could also be a mechanism to assure correct transcripts in the proliferative cells.

5.2.3 Transcription of at least three CMEs is altered at the onset of TSD (Paper III)

Using DNA microarray technique, the expression of genes coding for CMEs was assessed in NBK, SVpgC2a and SqCC/Y1 before and after three days of post-confluent culture with 5 % FBS. All 58 members of the ADH, ALDH, SDR, AKR and NQO (super-)families present on the Human Genome Focus Chip (contains 8400 genes) were evaluated in this study. Serum exposure for NBK resulted in more than two-fold altered expression of five CMEs, including increased expression of *ALDH1A3*, *DHRS3*,

HPGD, and *AKR1A1*, and decreased expression of *ALDH4A1* (Fig. 18). The transcriptional increase in response to serum was in part conserved in one or both of the transformed cell lines for *ALDH1A3* (4.4-fold increase in SVpgC2a) and *DHRS3* (2.8-fold increase in both SVpgC2a and SqCC/Y1). The fact that the addition of serum causes these genes to change expression substantially in SVpgC2a and SqCC/Y1 without causing them to differentiate highlights the possibility that these changes could be due to TSD-unrelated effects of serum. Hence, in line with the observed pattern for TSD markers, CME genes associated with serum-induced growth inhibition/TSD should typically be represented by transcriptional changes, which are observed in NBK upon serum exposure, but not in SVpgC2a and SqCC/Y1.

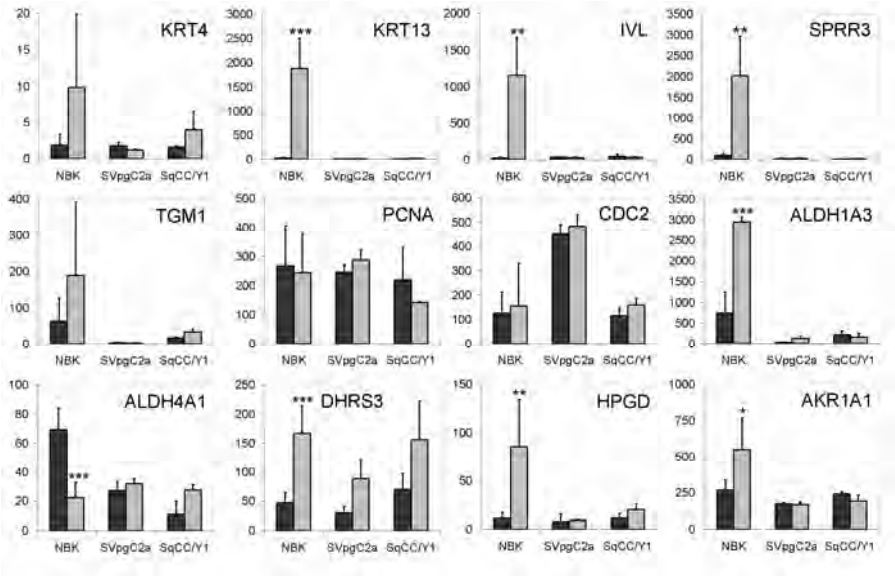


Fig. 18: Serum-responsive gene expression of suprabasal keratinocyte markers (*KRT4*, *KRT13*), markers of early *(IVL*, *SPRR3*) and late TSD (*TGM1*), proliferation markers (*PCNA*, *CDC2*) and significantly altered CMEs (*ALDH1A3*, *ALDH4A1*, *DHRS3*, *HPGD*, and *AKR1A1*). Dark-gray bars depict results for confluent cells; light-gray bars for post-confluent serum-exposed cells. Average signal intensities (y-values) are presented with standard deviations and derived from six hybridization experiments for NBK ± FBS and from two hybridization experiments for SVpgC2a and SqCC/Y1. Statistical analysis, in addition to the one provided by GCOS, was performed for NBK ± FBS by unpaired two-tailed t-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). All differentially transcribed CMEs were furthermore verified by Statistical Analysis of Microarray (SAM) [224].

Thus, the results suggest that altered transcription of at least three CME genes, *i.e. ALDH4A1*, *HPGD*, and *AKR1A1* is altered at the onset of TSD. Notably, these three

changes can tentatively be ascribed to signal transduction pathways with established or plausible function in keratinocyte differentiation. Transcription of p53-transduced *ALDH4A1* is lost in serum-differentiated NBK as well as in both transformed cell lines which might reflect p53-status in all three cases: p53 is inactivated in SVpgC2a, absent in SqCC/Y1 and p53 effects appear to be counteracted by p63 during keratinocyte differentiation [6, 77, 81, 225, 226]. *HPGD* is transduced by TGF-β, a TSD-inducing serum component, and considered to fulfill a tumor suppressor function in many cancer types [118, 227-229]. Finally, *AKR1A1* gene expression is among others regulated by CHOP (C/EBP homologous protein) which has been shown to be upregulated during early stages of TSD in keratinocytes [230, 231]. To date, no xenobiotic activity has been reported for ALDH4A1, a mitochondrial enzyme catalyzing the NAD^+-dependent second step in the degradation of proline to glutamate [232]. In contrast, HPGD is a COX-2 antagonist which, in addition to its function in the inactivation of prostaglandins, has been suggested to play a role in xenobiotic metabolism [114, 119]. Finally, *AKR1A1* codes for a ubiquitous aldehyde reductase which displays activity for both biogenic and exogenous aldehydes as well as anticancer drugs and quinones [131].

5.2.4 CME-mediated xenobiotic activity is increased at the onset of TSD (Paper III)

Taking advantage of the promiscuous substrates menadione and 9,10-PQ in combination with different relevant cofactors and inhibitors, efforts were made to assess CME-mediated xenobiotic activity in response to serum. NBK, but none of the transformed cell lines, exhibited increased menadione reduction activity in response to serum. The activity increase was characterized by dual NADPH/NADH specificity as well as high dicoumarol sensitivity, notably both properties of NQO1 [84]. Hence, the data is suggestive of NQO1 as the responsible enzyme.

NADPH-dependent 9,10-PQ reduction activity was increased in NBK and SqCC/Y1 in response to serum, but not in SVpgC2a. The activity increase was rutin-sensitive for NBK, but dicoumarol-sensitive for SqCC/Y1 which implies that the enzyme responsible for the activity increase in SqCC/Y1 is not the same as the one in NBK. The sensitivity towards rutin suggests increased AKR and/or CBR activity in serum-differentiated NBK [132, 135, 233, 234]. 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 translate to the activity increase observed in lysate from serum-exposed NBK (cf. Fig. 18) [131]. Apart from serum-exposed SqCC/Y1, dicoumarol did not have any effect on NADPH-dependent 9,10-PQ reduction activity. This implies that CRYZ does not contribute significantly to *ortho*-quinone metabolism in neither NBK nor SVpgC2a, and, with CRYZ transcripts being close to detection limit in SqCC/Y1, neither in SqCC/Y1 [101]. Finally, NADH-dependent 9,10-PQ reduction was less variable and, surprisingly, much more efficient in all cell lysates, resulting in two- to threefold higher activities. As no activity with prostaglandin F2α was detected, the NAD^+-dependent HPGD was unlikely to be responsible for this high activity [114].

Taken together, the results on quinone reduction activity suggest considerable capacity for CME-mediated xenobiotic metabolism in buccal epithelial cells which is significantly altered in the serum-differentiated state. Notably, to attribute these effects to specific CMEs, more directed approaches are needed including *e.g.* immuno-depletion or siRNA techniques.

5.3 THE DUAL FUNCTION OF ADH3 AS GSNO REDUCTASE AND FORMALDEHYDE DEHYDROGENASE

Table 3: A comparison of thus far reported steady-state kinetic parameters for GSNO reduction and HMGSH oxidation by human ADH3. k_{cat} values are calculated per ADH3 monomer.

HMGSH			GSNO			Reference
K_m [µM]	k_{cat} [min^{-1}]	k_{cat}/K_m [$min^{-1}µM^{-1}$]	K_m [µM]	k_{cat} [min^{-1}]	k_{cat}/K_m [$min^{-1}µM^{-1}$]	
0.12	150	1300	11.1	1,200	110	this thesis
2	115	58	27	2,400	90	[29]
1.7	150	90	4.8	1,760	370	[235]
1.4	160	115	27	6,000	220	[236]
0.63	56	89	n.d.	n.d.	n.d.	[157]
0.8	62	78	n.d.	n.d.	n.d.	[167]

Initially, steady-state kinetics for HMGSH oxidation and GSNO reduction was assessed by fluorescence spectroscopy, for better sensitivity as compared to the more

commonly used absorption spectroscopy. In this study, the K_m found for HMGSH was five- to tenfold lower than in previous studies, while the k_{cat} as well as the kinetic parameters for GSNO reduction were mostly consistent with previously reported values (Table 3).

5.3.1 GSNO promotes ADH3-mediated oxidative reactions and is concurrently reduced under cofactor recycling (Paper IV)

ADH3-mediated alcohol (octanol, ethanol and HMGSH) oxidation was monitored in absence and presence of GSNO by fluorescence and absorption spectroscopy, taking advantage of the fact that NADH and GSNO both absorb at 340 nm while only NADH fluoresces. With GSNO being a substrate for the reverse reaction, a product inhibition pattern would have been expected. However, in presence of GSNO an initial decrease in absorbance was observed which was attributed to the reduction of GSNO, as only NAD^+ had been added to the reaction mixture. Fluorescence spectroscopy showed that the initial negative slope coincided with absence of net NADH formation, indicating that NADH was immediately recycled by GSNO reduction (Fig. 19). Reaction velocities could thus be calculated in absence and presence of GSNO using the molar extinction coefficients of NADH (6220 $M^{-1}cm^{-1}$) and GSNO (840 $M^{-1}cm^{-1}$), respectively.

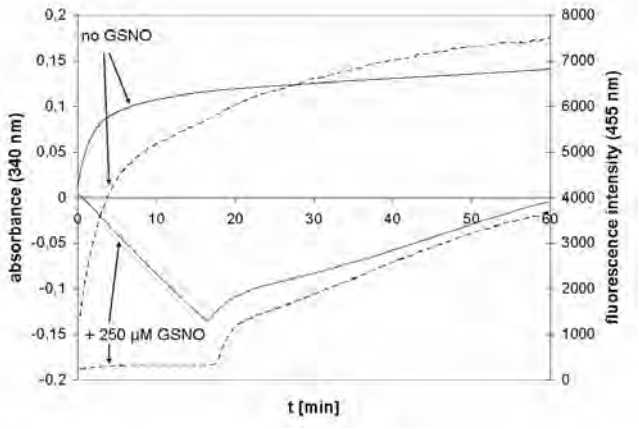


Fig. 19: Measurement principle of ADH3-catalyzed octanol oxidation in presence and absence of GSNO. Solid lines show reactions monitored by following absorbance at 340 nm. Dashed lines show reactions monitored by the NADH-specific fluorescence emission at 455 nm with 340 nm as excitation wavelength. In the presence of GSNO, the initial negative slope coincides with absence of net NADH production. Reactions were carried out in 0.1 M potassium phosphate buffer pH 7.5, 2.4 mM NAD^+, 2.0 mM octanol and 62.5 µg/ml ADH3 in absence and presence of 250 µM GSNO.

A comparison revealed that not only was GSNO reduction facilitated by ADH3-mediated alcohol oxidation providing the reduced cofactor, but the overall reaction rates were increased, notably up to eight-fold for the substrate pair HMGSH/GSNO. The results are consistent with immediate enzyme-bound cofactor recycling, as cofactor release is a step that is partially rate-limiting in HMGSH oxidation [235]. Strikingly, this effect was more pronounced when HMGSH oxidation was assessed with crude murine liver and lung lysates instead of purified ADH3. Here, presence of GSNO increased reaction rates about 20-fold. At the same time, GSNO degradation was very low in all controls without formaldehyde, excluding a significant contribution of non-enzymatic GSNO degradation to overall GSNO decay. Hence, circumvention of cofactor release apparently gains importance in a more complex environment including other NAD^+/NADH-scavenging enzymes. Notably, in presence of 1 mM ethanol instead of formaldehyde as substrate in these reactions, GSNO was still reduced, but no reaction rate increase was observed. At such low concentrations the contribution of ADH3 to ethanol oxidation is negligible, as ADH1 is the main ethanol-oxidizing enzyme in liver. Thus, in this case NADH could not be provided in ADH3-bound form and instead, the rate of GSNO reduction was limited by the rate of free NADH production following ADH1-mediated ethanol oxidation. Hence, through the proposed mechanism, enzyme-bound cofactor recycling, formaldehyde might induce rapid GSNO depletion under physiological conditions, including the typically high free NAD^+/NADH ratio (Fig. 20) [182]. It appears that any source of NADH can trigger GSNO reduction but that GSNO depletion is much more rapid, when the redox cycle is constrained to ADH3.

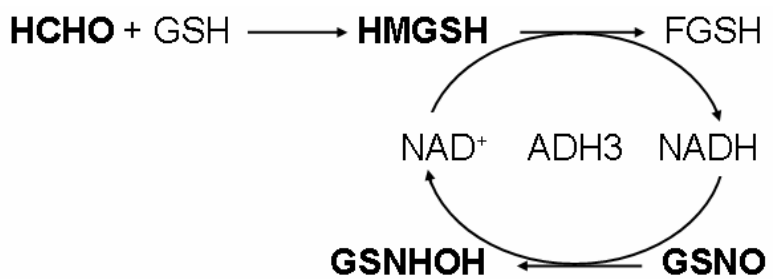


Fig. 20: Model for formaldehyde (HCHO)-induced GSNO depletion. The spontaneously formed adduct between HCHO and GSH, HMGSH, is oxidized by ADH3 under concomitant conversion of NAD^+ to NADH. The latter does not dissociate from the enzyme, but is directly used for GSNO reduction to the intermediate semimercaptal S-hydroxylaminoglutathione (GSNHOH).

5.3.2 Formaldehyde-triggered GSNO depletion, mediated by ADH3, might underlie the asthma-exacerbating effects of formaldehyde

It is highly striking that two reverse substrates of ADH3 that trigger or promote each others' conversion have opposite effects in asthma. Formaldehyde is known to act as a bronchoconstrictor and exacerbates asthma symptoms, although the molecular causes are not well understood [171-173]. In contrast, GSNO acts as an endogenous bronchodilator in airway lining fluid, protects from hyperresponsivity, and is depleted from airways of asthmatic patients [32, 197]. Evidence for an adverse role of ADH3 in asthma was provided by a study using Adh3-deficient mice which showed that genetic ablation of ADH3 results in higher levels of SNOs in lung homogenates and associates with protection from airway hyperresponsitivity [32]. Moreover, two single nucleotide polymorphisms in the *ADH3* gene have been coupled to asthma susceptibility [161]. A causal relationship between formaldehyde and decreased SNO levels in the regulation of airway responsivity, with ADH3 as the interconnecting factor, has been considered, but the findings presented above provide for the first time experimental evidence for one [237]. Accordingly, under asthmatic conditions, including lung epithelial cell damage, ADH3, GSH and NAD^+ are likely to be present in the airway lining fluid and thus, inhalation of formaldehyde might lead to rapid depletion of GSNO, resulting in bronchoconstriction and enhanced airway hyperresponsivity. In support of this hypothesis, it was shown in paper IV that exposure of cultured cells (SqCC/Y1) to formaldehyde results in a trend towards decreased SNO levels. Taken together, the presented results of paper IV are highly suggestive of an ADH3-driven mechanism underlying the well-established asthma-exacerbating properties of formaldehyde, inhalation of which would entail depletion of the protective bronchodilator GSNO. Nevertheless, the relevant experiments to prove that such a mechanism functions *in vivo* remain to be performed.

5.3.3 GSNO reduction results in the GSH-controlled formation of glutathione transferase (GST) inhibitors (Paper IV)

Product formation following GSNO reduction was assessed as a function of GSH concentrations. To this end, reaction mixtures were resolved on a strong anion exchange column after different reaction times, followed by (semi-)quantification by peak integration and qualitative reactant/product identification by ESI MS. The results showed equimolar consumption of NADH relative to GSNO, increasing hydroxylamine, decreasing glutathione sulfinamide formation in response to increasing

GSH concentrations, as well as glutathione disulfide as major reaction product at the highest GSH concentration. These findings support the results by Jensen *et al*. [28].

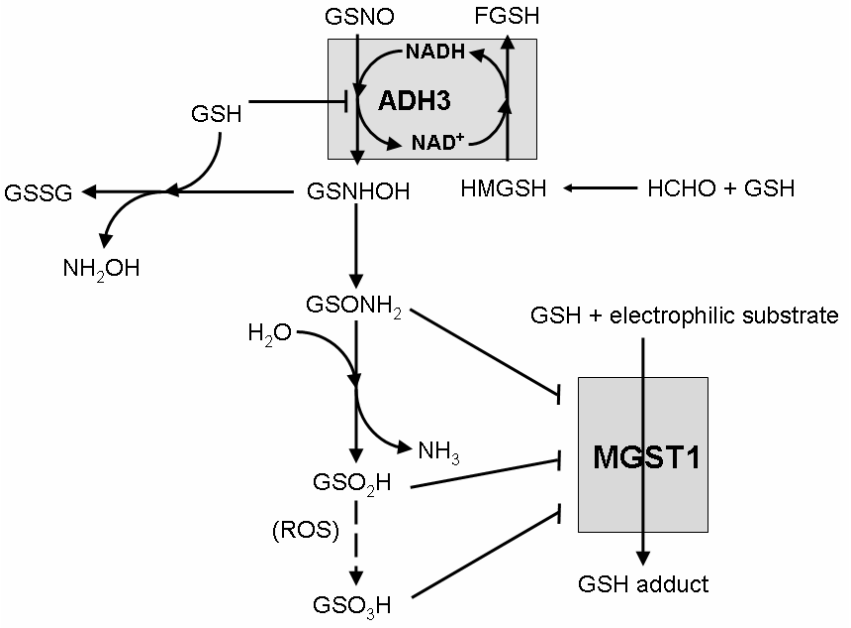


Fig. 21. Model for GSH-responsive product formation of ADH3-mediated GSNO reduction. In absence of GSH, ADH3-mediated GSNO reduction is fast and the intermediate S-hydroxylaminoglutathione can be spontaneously rearranged to the sulfinamide. Glutathione sulfinamide is partly hydrolyzed to the sulfinic acid which is likely to be oxidized to glutathionesulfonic acid under oxidative stress (ROS, reactive oxygen species). These three product species, glutathione sulfinamide, sulfinic acid and sulfonic acid, increasingly inhibit MGST1. In contrast, GSH at millimolar concentrations decreases the rate of ADH3-mediated GSNO reduction and the intermediate S-hydroxyaminoglutathione is intercepted by GSH to yield GSSG and NH_2OH. Invariably, NADH for GSNO reduction can be provided by oxidative ADH3 pathways, *e.g.* by oxidation of the glutathione adduct of formaldehyde, HMGSH.

While GSSG can be recycled to GSH by glutathione reductase and other enzymes, it was unclear whether glutathione sulfinamide, if produced under physiological conditions, would be metabolized further. Notably, in reactions with crude liver lysate instead of purified ADH3 a similar product formation pattern in response to GSH concentrations was observed, indicating that glutathione sulfinamide is a metabolic dead-end. The only product formed from glutathione sulfinamide that could be detected, generally after longer reaction times and at low GSH/GSNO ratios, was its spontaneous hydrolysis product glutathione sulfinic acid. As glutathionesulfonic acid is a well-known inhibitor for GSTs, it was assessed whether glutathione sulfinamide and

glutathione sulfinic acid equally inhibited MGST1 [143, 144]. Both compounds were found to inhibit MGST1 with IC_{50} values of 48 µM (sulfinamide) and 22 µM (sulfinic acid), close to the IC_{50} value of the established inhibitor glutathionesulfonic acid (5.3 µM) and considerably stronger than GSNO with an IC_{50} of 520 µM.

Taken together, the results imply that, under physiological conditions, GSNO reduction is decelerated by intracellular GSH (5-10 mM) which favors the interception of the intermediate semimercaptal by GSH and leads to the formation of GSSG as major product. However, severe oxidative stress, GSH depletion under disease conditions, or varying intracellular distribution of GSH might lead to the formation of the GST inhibitors glutathione sulfinamide and sulfinic acid and thus exacerbate toxic responses (Fig. 21) [238, 239].

5.3.4 Different substrate analogues as inhibitors of ADH3-mediated GSNO reduction (Paper V)

The above presented evidence suggests that ADH3 exhibits a Janus face. While ADH3 is known as a central player in the cellular protection against formaldehyde, it has now emerged that ADH3 might bring about adverse effects through its function as GSNO reductase, particularly evident in asthma [32]. First, ADH3 can lead to irreversible GSNO depletion if provided the appropriate cofactor NADH. Second, GSNO reduction can lead to the formation of GST inhibitors. In the light of these adverse effects, a specific ADH3 inhibitor might be of direct clinical use, particularly for the treatment of asthma, but possibly also for other disease conditions. Thus, a range of substrate analogues, including medium-chain fatty acids and GSH-derived compounds, was tested as inhibitors of ADH3-mediated GSNO reduction. Notably, of all known ADH3-substrates, the GSH-derived substrates HMGSH and GSNO display the highest affinity, as reflected by (sub-)micromolar K_m-values reported in paper IV and previous studies (cf. Table 3). Medium-chain ω-hydroxy fatty acids, particularly 10-hydroxydecanoic acid and 12-hydroxydodecanoic acid exhibit higher, but still micromolar, K_m values (paper V) [158, 240]. Hence, analogues for both substrate types were tested as inhibitors.

Irrespective of substrate analogue tested, with GSNO as variable substrate and a constant concentration of NADH, in most cases the model for non-competitive inhibition yielded the best data fit. In contrast, dodecanoic acid has previously been shown to competitively inhibit ADH3-mediated oxidative reactions with respect to

HMGSH and 12-hydroxydodecanoic acid reflecting that dodecanoic acid binds into the active site, as also evident from the crystal structure [166, 167, 235]. Notably, for alcohol dehydrogenase-catalyzed reactions, it is not uncommon for substrate analogues to show competitive inhibition in one reaction direction but uncompetitive or non-competitive inhibition in the reverse direction. This observation is frequently explained by the inhibitor's preference for binding one of the two possible enzyme-cofactor complexes (the enzyme-NAD^+ or the enzyme-NADH complex) [167, 241]. For the substrate analogues tested here, this suggests that the formation of enzyme-NAD^+-inhibitor complexes is favored over enzyme-NADH-inhibitor complexes.

Table 4. Medium-chain fatty acids as inhibitors for ADH3-mediated GSNO reduction.

fatty acid	$\mathbf{K_i}$ [μM][a]
octanoic (caprylic) acid	909 ± 69
nonanoic (pelargonic) acid	621 ± 63
decanoic (capric) acid	231 ± 11
undecanoic acid	190 ± 22
dodecanoic (lauric) acid	177 ± 26
tridecanoic acid	224 ± 6
decanedioic (sebacic) acid	437 ± 77
dodecanedioic acid	173 ± 18
2-aminodecanoic acid	NI[b]

[a] evaluated with the model for non-competitive inhibition

[b] no inhibition observed with 400 μM

The inhibition constants for fatty acids were in the micromolar range, higher than but close to the K_m-values of the corresponding substrates, and showed a clear dependency on chain length. The most efficient inhibitors were found to be undecanoic acid, dodecanoic acid and dodecanedioic acid, with no significant differences in inhibition constant (Table 4). Evident from the ADH3 structure with 12-hydroxydodecanoic acid, the binding of ω-hydroxy fatty acids relies on interactions of the carboxyl group with the anion binding pocket (Q111, R114, and K283 from the other subunit) at the base of the active site, interactions of the long aliphatic chain with

hydrophobic residues lining the active site towards the top, and finally the active site zinc [166]. Non-substituted fatty acids cannot interact favorably with the active site zinc as they are devoid of the nucleophilic group on the ω-carbon. Hence, in efforts to increase affinity by adding a zinc binding moiety on the ω-carbon of the inhibitor, two maximally oxidized substrate analogues, decanedioic acid and dodecanedioic acid, were tested as inhibitors. However, this did not improve affinity and actually had an unfavorable effect in the case of decanedioic acid. Furthermore, the introduction of an amino group in decanoic acid (2-aminodecanoic acid) abolished inhibition. Taken together, these results suggest that the active site, when binding fatty acids, can accommodate a second polar group at the ω-carbon if the chain length is optimized for maximal hydrophobic interactions, but overall binding affinity is thereby not improved.

Table 5. GSH derivatives as inhibitors for ADH3-mediated GSNO reduction.

GSH (derivative)	**K_i** [mM][a]
GSH	21 ± 3
S-methylglutathione	15 ± 1
S-acetamidoglutathione	6.1 ± 0.6
GSSG	> 50[b]
glutathione sulfonic acid	NI[c]

[a] evaluated with the model for non-competitive inhibition

[b] weak inhibition with 10 and 20 mM

[c] no inhibition observed with 10 mM

In contrast to the Michaelis constants for the high-affinity substrates HMGSH and GSNO, the K_i for GSH is in the millimolar range (papers IV and V, Table 5) [28]. The inhibitory effect was only marginally improved by substitution of the sulfhydryl proton with a methyl group. Hence, removal of the binding group to the active site zinc reduces affinity by three orders of magnitude for GSH-derived ligands. The addition of a potential zinc binding moiety to the glutathione scaffold was attempted by introducing an acetamido group, where either the carboxyl oxygen or the amino nitrogen is in theoretical zinc coordination distance. This improved inhibitory capacity threefold in comparison to GSH, but K_i was still in the millimolar range, possibly due to unfavorable sterical or electrostatical interactions emanating from the electronegative

atom that had remained uncoordinated to the zinc. Finally, very weak or no inhibition was observed with glutathione disulfide and glutathionesulfonic acid (Table 5).

Protein-ligand docking simulations for ADH3 using GSNO and S-methylglutathione (MGSH) as ligands were performed to address the considerable discrepancies in affinity. The results suggested that GSNO binds in a comparably well-ordered fashion in a manner similar to HMGSH. In contrast, the binding of MGSH was considerably less ordered and three independent docking simulations resulted in distinctly different ligand conformations including one where the ligand had escaped the active site. The difference in calculated binding free energies from the lowest-energy conformations of each docking simulation translated to by two magnitudes decreased dissociation constants which is qualitatively consistent with the experimental K_m and K_i values. Taken together, the findings imply that, for GSH-derived ADH3 ligands, a nucleophilic group in coordination distance of the active site zinc is imperative for efficient binding. This is in contrast to the binding of medium-chain fatty acid derivatives where the active site zinc plays an inferior role in binding and the addition of zinc-binding moieties does not improve overall binding affinity.

6 CONCLUSIONS

Assessment of differential gene expression in SVpgC2a and SqCC/Y1 relative to NBK by 2D-PAGE and DNA microarray analysis, assisted by selected bioinformatics tools, lead to the identification of multiple previously proposed and potential novel markers for BSCC. The markers included such at transcript and protein level as well as novel integrative markers as GO categories or aberrant molecular networks with their central regulator genes, representing potential tumor suppressor/promoter genes. A significant part of the single markers had previously been implied for buccal or oral carcinogenesis, supporting the overall approach. Hence, the culture of NBK, SVpgC2a and SqCC/Y1 provides a good model system for buccal carcinogenesis mechanisms, including for the testing of -omics data mining as novel diagnostic strategy. The methodology put forward contemplates the concept of multiple markers or aberrant molecular networks as more sensitive diagnostic and prognostic tools than single markers. Furthermore, the approach appears useful under circumstances of limited material, *e.g.* for individualized therapy.

Post-confluent culture in absence and presence of FBS was successfully used to model different *strata* of the buccal epithelium, as indicated by the CFE assay, changes in cell morphology and altered expression of established markers of proliferation and TSD. Notably, the overall results suggest that more than one growth protocol is required to mimic the complete buccal epithelium *in vitro*. Post-confluent culture of NBK for three days in presence of 5 % FBS appears to effectively capture the onset of TSD. As to CME transcription in association with proliferation and TSD, the results of paper II argued for a positive correlation of *ADH3* transcription with proliferation as opposed to a negative correlation with the process of TSD. In addition, collective assessment of CME gene expression indicated that transcription of three more CME genes, namely *ALDH4A1*, *AKR1A1* and *HPGD*, is altered at the onset of TSD. Enzymatic quinone reduction activities in crude cell lysates suggested considerable capacity for CME-mediated xenobiotic metabolism in NBK as well as increased NQO1 and AKR activity in post-confluent serum-differentiated NBK. Although the results need to be verified by *e.g.* immunodepletion or siRNA techniques, the presented work identified AKR1A1 and NQO1 as potential targets for neutralizable anti-neoplastic drugs in the treatment of BSCC.

The high cytosolic free NAD^+/NADH ratio does not favor reductive reactions that are dependent on NADH, and NADPH cannot efficiently be used by ADH3 [29, 182]. The results of paper IV showed that, *in vitro*, an unfavorable NAD^+/NADH ratio can be overcome in the presence of a substrate for an ADH3-mediated oxidative reaction by immediate enzyme-bound cofactor recycling. This was most efficiently achieved by formaldehyde in the form of its GSH adduct HMGSH, but in principle, this could be triggered by any source of NADH. Furthermore, formaldehyde exposure of SqCC/Y1 led to a significant decrease in total cellular SNO content. Taken together, the results provided indirect evidence for formaldehyde as a physiological trigger of GSNO depletion. The mechanism put forward offers a molecular explanation for the asthma-exacerbating effects of formaldehyde, where inhalation of formaldehyde leads to depletion of GSNO, an endogenous bronchodilator, in airway lining fluid.

Moreover, ADH3-mediated GSNO reduction can lead to the formation of glutathione sulfinamide and sulfinic acid, both potent inhibitors of GST activity. However, typical cellular GSH concentrations in the millimolar range decelerated the ADH3-catalyzed reaction and precluded the rearrangement to the sulfinamide by quantitative interception of the intermediate semimercaptale and formation of GSSG. Nevertheless, under conditions of low local GSH concentrations, *e.g.* following formaldehyde scavenging or under severe oxidative stress, toxic responses might be aggravated by the formation of GST inhibitors. The overall results imply that ADH3 exhibits a Janus face, as the major formaldehyde scavenger on the one hand, but with adverse effects due to its GSNO reductase activity on the other hand.

In the light of ADH3-mediated GSNO reduction as an adverse effect, various analogues of ADH3 substrates were tested as inhibitors for ADH3-mediated GSNO reduction. Among all tested inhibitors, the most efficient were undecanoic, dodecanoic acid and dodecanedioic acid. All GSH-derived inhibitors displayed inhibition constants in the millimolar range, at least three orders of magnitudes higher than the K_m values of the high-affinity substrates GSNO and HMGSH. The experimental results as well as docking simulations with GSNO and MGSH suggest that for ADH3 ligands with a glutathione scaffold a zinc-binding moiety is imperative for correct orientation and stabilization of the hydrophilic glutathione scaffold within a predominantly hydrophobic active site. The results provide guidelines for future design of a specific ADH3 inhibitor with potential direct clinical use for the prevention of asthma-exacerbating effects, and possibly for various other disease conditions.

7 ACKNOWLEDGEMENTS

This study was carried out at the division of Physiological Chemistry 1, Department of Medical Biochemistry and Biophysics, and the Unit of Biochemical Toxicology, Institute of Environmental Medicine, Karolinska Institutet. I wish to express my sincere gratitude to the following people for all kinds of help and support:

My main supervisor Professor **Jan-Olov Höög**, for accepting me as a PhD student. Thank you for your trust and the opportunity to work independently, but also for being available when necessary, especially during these last crazy months.

My co-supervisor Professor **Roland Grafström**, for bringing me here, for introducing me into the curious world of cell culture and for an astonishing ability to turn just about everything into something positive.

Professor **Hans Jörnvall**, for excellent working facilities, for great trips to Tallinn and Reykjavík, for the occasional birding report during Monday meetings and for support in ornithological and other scientific issues. Last, but certainly not least, both **Hans** and **Ann-Margaret Jörnvall** for "twitching" at its finest in Uppland/Sörmland.

Professor emeritus **Jan Sjövall**, for always listening with genuine interest, for support in various questions including, but not exclusively, scientific, and for a great sense of humor. I will miss our lunches. HEJ-Lab would not be the same without you!

Professor **Ralf Morgenstern**, for a great collaboration, for at all times being available for questions and discussions, and for being generous with ideas.

Rebecca Ceder, we are synergy! Thank you for being so much fun to work with! For sharing knowledge on methods and invaluable help in the lab: **Margareta Brandt**, **Jesper Hedberg**, **Mikko Hellgren**, **Theres Jägerbrink**, **Johan Lengqvist**, **Marina Merne**, **Jan-Anders Nilsson**, **Åke Norberg**, **Karin Roberg**, **Martin Vondracek**, and **Johan Ålander**. For kind permission to use equipment, Professor **Birgitta Agerberth**, for being a never-ceasing source of fatty acids and other organic chemicals, Professor **Mats Hamberg**.

Past and present HEJ-LAB-PhD students & Co. for "girly" activities (I won't specify), "manly" activities (beer-drinking, bowling) and, most importantly, friendship: **Rebecca Ceder**, **Andreas Cederlund**, **Michael Fitzen, Mikko Hellgren**, **Theres Jägerbrink**, **Ylva Kai-Larsen**, **Luise Landreh**, **Emma Lindahl**, **Uzma Naseeb**, **Maria Tollin**, and **Susanne Vollmer**. Good luck for the great things that lie ahead of you! **Theres**, thanks for always being available for spontaneous after-work beers; **Susanne**, thanks for patient ears, walks and talks, German lunches, compassion…

Lunchgängets kärna: **Tomas Bergman**, **Ella Cederlund**, **Carina Palmberg**, and **Jan Sjövall**, for great fun during lunches and for always showing (or faking) interest in bird-related subjects.

Roommates of the late months: **Michael Fitzen**, **Emma Lindahl**, **Uzma Naseeb** and **Essam Refai**; thanks for being so understanding and cutting down on the nut-eating (you really drove me nuts, Essam!), perfuming, chatting etc.; I really really appreciate it! ☺

A special thanks to **Michael** and **Luise** for proofreading my thesis before and after much-needed shortening...

The HEJ-Lab technicians, **Gunvor Alvelius**, **Margareta Brandt**, **Ella Cederlund**, **Monica Lindh**, **Carina Palmberg, Marie Ståhlberg,** and secretary emerita **Ingegerd Nylander:** It is true: We PhD students are the flow-through while you are the priceless column resin. Good luck to you for all future changes in Chemistry 1 and PAC!

Further past and present members of HEJ-Lab: My mentor **Magnus Gustafsson**, **Andreas Almlén**, **Peter Bergman**, **Gudmundur Bergsson**, **Karl Bodin**, **Erik Brandt**, **Haneen Hamada**, **Mikael Henriksson**, **Daniel Hirschberg**, **Malin Hult**, **Moin Ishrat**, **Jing Li**, **Ermias Melles**, **Udo Oppermann**, **Jenny Samskog**, **Protim Sarker**, **Jawed Shafqat**, **Naeem Shafqat**, **Rannar Sillard**, **Johnny Söderlund**, **Sam Tryggvason**, **Jan Wiberg**, **Xiaoqiu Wu,** and **Linus Östberg**.

MBB teaching staff: **Susie Björkholm**, Professor **Tomas Cronholm**, **Agneta Nordberg**, **Annika Pettersson, Susanne Rothstein**, for excellent assistance in teaching matters; **Åke Rökaeus**, for giving me teaching assignments that I could relate to - teaching was great fun!

For technical help and other assistance, **Angelica Arribada**, **Svante Backlund**, **Jan Hallensjö**, **Sören Lundmark**, and **Anders Lundsjö**.

Previous teachers for inspiration and support: Professor **Franz-Xaver Schmid** from the University of Bayreuth; Professor **Mathias Springer** and **Joël Caillet** from the *Institut de Biologie Physico-Chimique*, Paris.

For organizing a great meeting in Kranjska Gora, Slovenia, Professor **Henry Weiner** and **Esther Weiner**.

Olika grupper och föreningar för mycket uppskattad avkoppling av olika slag: Sånggruppen Soul Special, Ukulelekommissionen, Närnaturguiderna, SOF:s fågelintressekommitté, Stockholms betongskådare och Sveriges fåglar.

Freunde aus dem unterfränkischen/hessischen Grenzgebiet **Angi** und **Markus, Heike** und **Rocco, Anke** und **Thomas**, Schwägerin **Christiane**, Schwabe **Thomas K.**, und Ex-Bayreuther **Luci**, **Christoph**, **Meike**, **Silke**, **Insa** und **Michael** - danke für Besuche in meinem nördlichen Wahlexil! **Anne-Line** und **Martin, Anja** und **Jochen**, fürs Organisieren von Festen im schönen und praktisch gelegenen Moseltal. **Gerrit** und **Kersten**, dafür dass mit Euch immer in Traben-Trarbach zu rechnen ist. ☺

Lasse och **Ragnhild**, **Pelle**, **Klara**, **Ida** och **Norma**, för roliga familjeutflykter, fika, middagar, barnvaktstillfällen, semestrar i Toulon och Visby och och och...

Mama Staab für das erfolgreiche Führen des Hotels „Staab Breunsberg“ - Urlaub zuhause ist immer Erholung pur! **Papa Staab** für den Leitspruch ”Net nochlosse mecht die Abbeid fäddich.“ In Familie Staab wird nämlich höchstens der Flaschenöffner an den Nagel gehängt (damit im Garten neben den Blumen auch die Biere aufgehen). Den besten Geschwistern der Welt, **Susanne** (Thuthi) und **Burkhard**, dafür, dass Ihr mich immer mental unterstützt.

Anders W., ständig medobservatör, teknisk rådgivare, SOF-volontär-inhoppare, terapeut, bästa vän, och mycket, mycket mer - för att du har trott på mig, peppat mig, stöttat mig och ställt upp för mig på tusen olika sätt. TACK! Du är bäst! Nej, DU.

8 REFERENCES

1 Diaz, E. M., Jr., Holsinger, F. C., Zuniga, E. R., Roberts, D. B. and Sorensen, D. M. (2003) Squamous cell carcinoma of the buccal mucosa: one institution's experience with 119 previously untreated patients. Head Neck **25**, 267-73

2 Lin, C. S., Jen, Y. M., Cheng, M. F., Lin, Y. S., Su, W. F., Hwang, J. M., Chang, L. P., Chao, H. L., Liu, D. W., Lin, H. Y. and Shum, W. Y. (2006) Squamous cell carcinoma of the buccal mucosa: an aggressive cancer requiring multimodality treatment. Head Neck **28**, 150-7

3 Reibel, J. (2003) Prognosis of oral pre-malignant lesions: significance of clinical, histopathological, and molecular biological characteristics. Crit. Rev. Oral Biol. Med. **14**, 47-62

4 Hunter, K. D., Parkinson, E. K. and Harrison, P. R. (2005) Profiling early head and neck cancer. Nature Reviews Cancer **5**, 127-35

5 Voorzanger-Rousselot, N. and Garnero, P. (2007) Biochemical markers in oncology. Part I: molecular basis. Part II: clinical uses. Cancer Treat. Rev. **33**, 230-83

6 Reiss, M., Brash, D. E., Munoz-Antonia, T., Simon, J. A., Ziegler, A., Vellucci, V. F. and Zhou, Z. L. (1992) Status of the p53 tumor suppressor gene in human squamous carcinoma cell lines. Oncol. Res. **4**, 349-57

7 Ceder, R., Merne, M., Staab, C. A., Nilsson, J. A., Höög, J. O., Dressler, D., Engelhart, K. and Grafström, R. C. (2007) The application of normal, SV40 T-antigen-immortalised and tumour-derived oral keratinocytes, under serum-free conditions, to the study of the probability of cancer progression as a result of environmental exposure to chemicals. Altern. Lab. Anim. **35**, 621-39

8 Staab, C. A., Vondracek, M., Custodio, H., Johansson, K., Nilsson, J. A., Morgan, P., Höög, J. O., Cotgreave, I. and Grafström, R. C. (2004) Modelling of normal and premalignant oral tissue by using the immortalised cell line, SVpgC2a: a review of the value of the model. Altern. Lab. Anim. **32**, 401-5

9 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**, 331-40

10 Hansson, A., Bloor, B. K., Haig, Y., Morgan, P. R., Ekstrand, J. and Grafström, R. C. (2001) Expression of keratins in normal, immortalized and malignant oral epithelia in organotypic culture. Oral Oncol. **37**, 419-30

11 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**, 562-8

12 Grafström, R. C. (2002) in Culture of Epithelial Cells (Freshney, I. R. and Freshney, M. G., eds.), pp. 195-255, John Wiley and Sons, Inc., New York

13 Wertz, P. W. and Squier, C. A. (1991) Cellular and molecular basis of barrier function in oral epithelium. Crit. Rev. Ther. Drug Carrier Syst. **8**, 237-69

14 Shojaei, A. H. (1998) Buccal mucosa as a route for systemic drug delivery: a review. J. Pharm. Pharmacol. **1**, 15-30

15 Donato, M. T., Lahoz, A., Castell, J. V. and Gomez-Lechon, M. J. (2008) Cell lines: a tool for in vitro drug metabolism studies. Curr. Drug Metab. **9**, 1-11

16 Oppermann, U. C. and Maser, E. (2000) Molecular and structural aspects of xenobiotic carbonyl metabolizing enzymes. Role of reductases and dehydrogenases in xenobiotic phase I reactions. Toxicology **144**, 71-81

17 Jörnvall, H. (1994) The alcohol dehydrogenase system. EXS **71**, 221-9

18 Atalla, A., Breyer-Pfaff, U. and Maser, E. (2000) Purification and characterization of oxidoreductases-catalyzing carbonyl reduction of the tobacco-specific nitrosamine 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) in human liver cytosol. Xenobiotica **30**, 755-69

19 Novotna, R., Wsol, V., Xiong, G. and Maser, E. (2008) Inactivation of the anticancer drugs doxorubicin and oracin by aldo-keto reductase (AKR) 1C3. Toxicol. Lett. **181**, 1-6
20 Kassner, N., Huse, K., Martin, H. J., Godtel-Armbrust, U., Metzger, A., Meineke, I., Brockmoller, J., Klein, K., Zanger, U. M., Maser, E. and Wojnowski, L. (2008) CBR1 is a predominant doxorubicin reductase in the human liver. Drug Metab. Dispos. **36**, 2113-2120
21 Park, J. H., Mangal, D., Tacka, K. A., Quinn, A. M., Harvey, R. G., Blair, I. A. and Penning, T. M. (2008) Evidence for the aldo-keto reductase pathway of polycyclic aromatic trans-dihydrodiol activation in human lung A549 cells. Proc. Natl. Acad. Sci. U. S. A. **105**, 6846-51
22 Duester, G., Mic, F. A. and Molotkov, A. (2003) Cytosolic retinoid dehydrogenases govern ubiquitous metabolism of retinol to retinaldehyde followed by tissue-specific metabolism to retinoic acid. Chem.-Biol. Interact. **143-144**, 201-10
23 Oppermann, U. (2007) Carbonyl reductases: the complex relationships of mammalian carbonyl- and quinone-reducing enzymes and their role in physiology. Annu. Rev. Pharmacol. Toxicol. **47**, 293-322
24 IARC. (2005) Monograph: Evaluation of carcinogenic risks to humans. Formaldehyde, 2-butoxyethanol and 1-tert-butoxy-2-propanol. IARC Monogr. Eval. Carcinog. Risks Hum. **88**
25 Holmquist, B. and Vallee, B. L. (1991) Human liver class III alcohol and glutathione dependent formaldehyde dehydrogenase are the same enzyme. Biochem. Biophys. Res. Commun. **178**, 1371-7
26 Koivusalo, M., Baumann, M. and Uotila, L. (1989) Evidence for the identity of glutathione-dependent formaldehyde dehydrogenase and class III alcohol dehydrogenase. FEBS Lett. **257**, 105-9
27 Hedberg, J. J., Hoog, J. O., Nilsson, J. A., Xi, Z., Elfwing, A. and Grafstrom, R. C. (2000) Expression of alcohol dehydrogenase 3 in tissue and cultured cells from human oral mucosa. Am. J. Pathol. **157**, 1745-55
28 Jensen, D. E., Belka, G. K. and Du Bois, G. C. (1998) S-Nitrosoglutathione is a substrate for rat alcohol dehydrogenase class III isoenzyme. Biochem. J. **331 (Pt 2)**, 659-68
29 Hedberg, J. J., Griffiths, W. J., Nilsson, S. J. and Hoog, 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**, 1249-56
30 Haqqani, A. S., Do, S. K. and Birnboim, H. C. (2003) The role of a formaldehyde dehydrogenase-glutathione pathway in protein S-nitrosation in mammalian cells. Nitric Oxide **9**, 172-81
31 Liu, L., Hausladen, A., Zeng, M., Que, L., Heitman, J. and Stamler, J. S. (2001) A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. Nature **410**, 490-4
32 Que, L. G., Liu, L., Yan, Y., Whitehead, G. S., Gavett, S. H., Schwartz, D. A. and Stamler, J. S. (2005) Protection from experimental asthma by an endogenous bronchodilator. Science **308**, 1618-21
33 Hoffmann, F. and Maser, E. (2007) Carbonyl reductases and pluripotent hydroxysteroid dehydrogenases of the short-chain dehydrogenase/reductase superfamily. Drug Metab. Rev. **39**, 87-144
34 Squier, C. A. and Kremer, M. J. (2001) Biology of oral mucosa and esophagus. J. Natl. Cancer Inst. Monogr., 7-15
35 Harris, D. and Robinson, J. R. (1992) Drug delivery via the mucous membranes of the oral cavity. J. Pharm. Sci. **81**, 1-10
36 Yurchenco, P. D. and O'Rear, J. J. (1994) Basal lamina assembly. Curr. Opin. Cell Biol. **6**, 674-81
37 Presland, R. B. and Jurevic, R. J. (2002) Making sense of the epithelial barrier: what molecular biology and genetics tell us about the functions of oral mucosal and epidermal tissues. J. Dent. Educ. **66**, 564-74

38 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
39 Morgan, P. R. and Su, L. (1994) Intermediate filaments in oral neoplasia. I. Oral cancer and epithelial dysplasia. Eur. J. Cancer. B. Oral Oncol. **30B**, 160-6
40 Fradette, J., Germain, L., Seshaiah, P. and Coulombe, P. A. (1998) The type I keratin 19 possesses distinct and context-dependent assembly properties. J. Biol. Chem. **273**, 35176-84
41 Katou, F., Shirai, N., Kamakura, S., Tagami, H., Nagura, H. and Motegi, K. (2003) Differential expression of cornified envelope precursors in normal skin, intraorally transplanted skin and normal oral mucosa. Br. J. Dermatol. **148**, 898-905
42 Eckert, R. L. and Welter, J. F. (1996) Epidermal keratinocytes - genes and their regulation. Cell Death Differ. **3**, 373-83
43 Kautsky, M. B., Fleckman, P. and Dale, B. A. (1995) Retinoic acid regulates oral epithelial differentiation by two mechanisms. J. Invest. Dermatol. **104**, 546-53
44 Isseroff, R. R., Chun, K. T. and Rosenberg, R. M. (1989) Triiodothyronine alters the cornification of cultured human keratinocytes. Br. J. Dermatol. **120**, 503-10
45 Pillai, S., Bikle, D. D. and Elias, P. M. (1988) 1,25-Dihydroxyvitamin D production and receptor binding in human keratinocytes varies with differentiation. J. Biol. Chem. **263**, 5390-5
46 Stojadinovic, O., Lee, B., Vouthounis, C., Vukelic, S., Pastar, I., Blumenberg, M., Brem, H. and Tomic-Canic, M. (2007) Novel genomic effects of glucocorticoids in epidermal keratinocytes: inhibition of apoptosis, interferon-gamma pathway, and wound healing along with promotion of terminal differentiation. J. Biol. Chem. **282**, 4021-34
47 Menon, G. K., Grayson, S. and Elias, P. M. (1985) Ionic calcium reservoirs in mammalian epidermis: ultrastructural localization by ion-capture cytochemistry. J. Invest. Dermatol. **84**, 508-12
48 Parkin, D. M., Bray, F., Ferlay, J. and Pisani, P. (2005) Global cancer statistics, 2002. CA. Cancer J. Clin. **55**, 74-108
49 Ostman, J., Anneroth, G., Gustafsson, H. and Tavelin, B. (1995) Malignant oral tumours in Sweden 1960-1989--an epidemiological study. Eur. J. Cancer. B. Oral Oncol. **31B**, 106-12
50 Muir, C. and Weiland, L. (1995) Upper aerodigestive tract cancers. Cancer **75**, 147-53
51 Moore, S. R., Johnson, N. W., Pierce, A. M. and Wilson, D. F. (2000) The epidemiology of mouth cancer: a review of global incidence. Oral Dis. **6**, 65-74
52 Fang, F. M., Leung, S. W., Huang, C. C., Liu, Y. T., Wang, C. J., Chen, H. C., Sun, L. M. and Huang, D. T. (1997) Combined-modality therapy for squamous carcinoma of the buccal mucosa: treatment results and prognostic factors. Head Neck **19**, 506-12
53 IARC. (1985) Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans: Tobacco habits other than smoking; Betel-quid and areca nut chewing; and some related nitrosamines. IARC Monogr. Eval. Carcinog. Risks Hum. **37**
54 IARC. (2004) Betel-quid and Areca-nut Chewing and Some Areca-nut-derived Nitrosamines. IARC Monogr. Eval. Carcinog. Risks Hum. **85**
55 Ko, Y. C., Huang, Y. L., Lee, C. H., Chen, M. J., Lin, L. M. and Tsai, C. C. (1995) Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. J. Oral Pathol. Med. **24**, 450-3
56 Walker, D. M., Boey, G. and McDonald, L. A. (2003) The pathology of oral cancer. Pathology (Phila). **35**, 376-83
57 Campisi, G., Panzarella, V., Giuliani, M., Lajolo, C., Di Fede, O., Falaschini, S., Di Liberto, C., Scully, C. and Lo Muzio, L. (2007) Human papillomavirus: its identity and controversial role in oral oncogenesis, premalignant and malignant lesions (review). Int. J. Oncol. **30**, 813-23

58 Nair, S. and Pillai, M. R. (2005) Human papillomavirus and disease mechanisms: relevance to oral and cervical cancers. Oral Dis. **11**, 350-9
59 Silverman, S. and Eversole, L. R. (2002) in Essentials of Oral Medicine (Silverman, S., Eversole, L. and Truelove, E., eds.), B C Decker Inc., Hamilton ON
60 Driemel, O., Kunkel, M., Hullmann, M., von Eggeling, F., Müller-Richter, U., Kosmehl, H. and Reichert, T. E. (2007) Diagnosis of oral squamous cell carcinoma and its precursor lesions. J. Dtsch. Dermatol. Ges. **5**, 1095-100
61 Schliephake, H. (2003) Prognostic relevance of molecular markers of oral cancer--a review. Int. J. Oral Maxillofac. Surg. **32**, 233-45
62 Lothaire, P., de Azambuja, E., Dequanter, D., Lalami, Y., Sotiriou, C., Andry, G., Castro, G., Jr. and Awada, A. (2006) Molecular markers of head and neck squamous cell carcinoma: promising signs in need of prospective evaluation. Head Neck **28**, 256-69
63 Nagpal, J. K. and Das, B. R. (2003) Oral cancer: reviewing the present understanding of its molecular mechanism and exploring the future directions for its effective management. Oral Oncol. **39**, 213-21
64 Otero-Rey, E., Garcia-Garcia, A., Barros-Angueira, F., Torres-Espanol, M., Gandara-Rey, J. M. and Somoza-Martin, M. (2004) DNA microarrays in oral cancer. Med. Oral **9**, 288-92
65 Massano, J., Regateiro, F. S., Januario, G. and Ferreira, A. (2006) Oral squamous cell carcinoma: review of prognostic and predictive factors. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. **102**, 67-76
66 Wrangle, J. M. and Khuri, F. R. (2007) Chemoprevention of squamous cell carcinoma of the head and neck. Curr. Opin. Oncol. **19**, 180-7
67 Scheer, M., Kuebler, A. C. and Zoller, J. E. (2004) Chemoprevention of oral squamous cell carcinomas. Onkologie **27**, 187-93
68 Brockstein, B. and Masters, G. (2002) in Cancer Treat. Res. (Rosen, S. T., ed.), Kluwer Academic Publishers, Secaucus, NJ
69 Ayllon Barbellido, S., Campo Trapero, J., Cano Sanchez, J., Perea Garcia, M. A., Escudero Castano, N. and Bascones Martinez, A. (2008) Gene therapy in the management of oral cancer: review of the literature. Med. Oral Patol. Oral Cir. Bucal **13**, E15-21
70 Choi, S. and Myers, J. N. (2008) Molecular pathogenesis of oral squamous cell carcinoma: implications for therapy. J. Dent. Res. **87**, 14-32
71 Gimenez-Conti, I. B. and Slaga, T. J. (1993) The hamster cheek pouch carcinogenesis model. J. Cell. Biochem. Suppl. **17F**, 83-90
72 Grafström, R. C., Noren, U. G., Zheng, X., Elfwing, Å. and Sundqvist, K. (1997) Growth and transformation of human oral epithelium in vitro. Recent Results in Cancer Research **143**, 275-306
73 Scully, C. (2002) Oral squamous cell carcinoma; from an hypothesis about a virus, to concern about possible sexual transmission. Oral Oncol. **38**, 227-34
74 Wise-Draper, T. M. and Wells, S. I. (2008) Papillomavirus E6 and E7 proteins and their cellular targets. Front. Biosci. **13**, 1003-17
75 Tsantoulis, P. K., Kastrinakis, N. G., Tourvas, A. D., Laskaris, G. and Gorgoulis, V. G. (2007) Advances in the biology of oral cancer. Oral Oncol. **43**, 523-34
76 Poulin, D. L. and DeCaprio, J. A. (2006) Is there a role for SV40 in human cancer? J. Clin. Oncol. **24**, 4356-65
77 Kulkarni, P. S., Sundqvist, K., Betsholtz, C., Höglund, P., Wiman, K. G., Zhivotovsky, B., Bertolero, F., Liu, Y. and Grafström, R. C. (1995) Characterization of human buccal epithelial cells transfected with the simian virus 40 T-antigen gene. Carcinogenesis **16**, 2515-21
78 Sacks, P. G. (1996) Cell, tissue and organ culture as in vitro models to study the biology of squamous cell carcinomas of the head and neck. Cancer Metastasis Rev. **15**, 27-51
79 Edington, K. G. (2004) in Culture of human tumor cells (Freshney, R. I. and Pfragner, R., eds.), pp. 261-288, John Wiley & Sons Inc, New Jersey

80 Chang, S. E., Foster, S., Betts, D. and Marnock, W. E. (1992) DOK, a cell line established from human dysplastic oral mucosa, shows a partially transformed non-malignant phenotype. Int. J. Cancer **52**, 896-902
81 Lippens, S., Denecker, G., Ovaere, P., Vandenabeele, P. and Declercq, W. (2005) Death penalty for keratinocytes: apoptosis versus cornification. Cell Death Differ. **12 Suppl 2**, 1497-508
82 Hansson, A., Bloor, B. K., Sarang, Z., Haig, Y., Morgan, P. R., Stark, H. J., Fusenig, N. E., Ekstrand, J. and Grafström, R. C. (2003) Analysis of proliferation, apoptosis and keratin expression in cultured normal and immortalized human buccal keratinocytes. Eur. J. Oral Sci. **111**, 34-41
83 Kulkarni Schmidt-Kastner, P. S., Heiden, T., Tribukait, B. and Grafström, R. C. (1996) Non-tumorigenic SV40T immortalized human buccal epithelial cells show aneuploidy and genetic instability. Anticancer Res. **16**, 2681-6
84 Lind, C., Cadenas, E., Hochstein, P. and Ernster, L. (1990) DT-diaphorase: purification, properties, and function. Methods Enzymol. **186**, 287-301
85 Wermuth, B. (1981) Purification and properties of an NADPH-dependent carbonyl reductase from human brain. Relationship to prostaglandin 9-ketoreductase and xenobiotic ketone reductase. J. Biol. Chem. **256**, 1206-13
86 Timbrell, J. A. (1999) Principles of Biochemical Toxicology, CRC Press, London, U.K.
87 Silbernagl, S. and Heuner, A. (1990) Renal transport and metabolism of mercapturic acids and their precursors. Toxicol. Lett. **53**, 45-51
88 Colucci, M. A., Moody, C. J. and Couch, G. D. (2008) Natural and synthetic quinones and their reduction by the quinone reductase enzyme NQO1: from synthetic organic chemistry to compounds with anticancer potential. Org. Biomol. Chem. **6**, 637-56
89 Atalla, A. and Maser, E. (1999) Carbonyl reduction of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in cytosol of mouse liver and lung. Toxicology **139**, 155-66
90 Maser, E. (1995) Xenobiotic carbonyl reduction and physiological steroid oxidoreduction. The pluripotency of several hydroxysteroid dehydrogenases. Biochem. Pharmacol. **49**, 421-40
91 Höög, J. O., Hedberg, J. J., Strömberg, P. and Svensson, S. (2001) Mammalian alcohol dehydrogenase - functional and structural implications. J. Biomed. Sci. **8**, 71-6
92 Matsunaga, T., Shintani, S. and Hara, A. (2006) Multiplicity of mammalian reductases for xenobiotic carbonyl compounds. Drug Metab. Pharmacokinet. **21**, 1-18
93 Jin, Y. and Penning, T. M. (2007) Aldo-keto reductases and bioactivation/detoxication. Annu. Rev. Pharmacol. Toxicol. **47**, 263-92
94 Cho, H., Oliveira, M. A. and Tai, H. H. (2003) Critical residues for the coenzyme specificity of NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase. Arch. Biochem. Biophys. **419**, 139-46
95 Di Luccio, E., Elling, R. A. and Wilson, D. K. (2006) Identification of a novel NADH-specific aldo-keto reductase using sequence and structural homologies. Biochem. J. **400**, 105-14
96 Wu, K., Knox, R., Sun, X. Z., Joseph, P., Jaiswal, A. K., Zhang, D., Deng, P. S. and Chen, S. (1997) Catalytic properties of NAD(P)H:quinone oxidoreductase-2 (NQO2), a dihydronicotinamide riboside dependent oxidoreductase. Arch. Biochem. Biophys. **347**, 221-8
97 Brändén, C. I., Jörnvall, H., Eklund, H. and Furugren, B. (1975) in The Enzymes, vol. 11 (Boyer, P. D., ed.), pp. 103-190, Academic Press, New York
98 Jörnvall, H., Höög, J. O., Persson, B. and Parés, X. (2000) Pharmacogenetics of the alcohol dehydrogenase system. Pharmacology **61**, 184-91
99 Yang, Z. N., Davis, G. J., Hurley, T. D., Stone, C. L., Li, T. K. and Bosron, W. F. (1994) Catalytic efficiency of human alcohol dehydrogenases for retinol oxidation and retinal reduction. Alcohol. Clin. Exp. Res. **18**, 587-91
100 Deltour, L., Foglio, M. H. and Duester, G. (1999) Metabolic deficiencies in alcohol dehydrogenase Adh1, Adh3, and Adh4 null mutant mice. Overlapping

roles of Adh1 and Adh4 in ethanol clearance and metabolism of retinol to retinoic acid. J. Biol. Chem. **274**, 16796-801
101 Rao, P. V., Krishna, C. M. and Zigler, J. S., Jr. (1992) Identification and characterization of the enzymatic activity of ζ-crystallin from guinea pig lens. A novel NADPH:quinone oxidoreductase. J. Biol. Chem. **267**, 96-102
102 Tang, A. and Curthoys, N. P. (2001) Identification of ζ-crystallin/ NADPH:quinone reductase as a renal glutaminase mRNA pH response element-binding protein. J. Biol. Chem. **276**, 21375-80
103 Nordling, E., Jörnvall, H. and Persson, B. (2002) Medium-chain dehydrogenases/reductases (MDR). Family characterizations including genome comparisons and active site modeling. Eur. J. Biochem. **269**, 4267-76
104 Fernández, M. R., Porté, S., Crosas, E., Barberà, N., Farrés, J., Biosca, J. A. and Parés, X. (2007) Human and yeast ζ-crystallins bind AU-rich elements in RNA. Cell. Mol. Life Sci. **64**, 1419-27
105 Vasiliou, V. and Pappa, A. (2000) Polymorphisms of human aldehyde dehydrogenases. Consequences for drug metabolism and disease. Pharmacology **61**, 192-8
106 Yoshida, A., Rzhetsky, A., Hsu, L. C. and Chang, C. (1998) Human aldehyde dehydrogenase gene family. Eur. J. Biochem. **251**, 549-57
107 Lindahl, R. and Petersen, D. R. (1991) Lipid aldehyde oxidation as a physiological role for class 3 aldehyde dehydrogenases. Biochem. Pharmacol. **41**, 1583-7
108 Mills, P. B., Struys, E., Jakobs, C., Plecko, B., Baxter, P., Baumgartner, M., Willemsen, M. A., Omran, H., Tacke, U., Uhlenberg, B., Weschke, B. and Clayton, P. T. (2006) Mutations in antiquitin in individuals with pyridoxine-dependent seizures. Nat. Med. **12**, 307-9
109 Pappa, A., Chen, C., Koutalos, Y., Townsend, A. J. and Vasiliou, V. (2003) Aldh3a1 protects human corneal epithelial cells from ultraviolet- and 4-hydroxy-2-nonenal-induced oxidative damage. Free Radic. Biol. Med. **34**, 1178-89
110 Marchitti, S. A., Orlicky, D. J. and Vasiliou, V. (2007) Expression and initial characterization of human ALDH3B1. Biochem. Biophys. Res. Commun. **356**, 792-8
111 O'Brien, P. J., Siraki, A. G. and Shangari, N. (2005) Aldehyde sources, metabolism, molecular toxicity mechanisms, and possible effects on human health. Crit. Rev. Toxicol. **35**, 609-62
112 Lindahl, R. (1992) Aldehyde dehydrogenases and their role in carcinogenesis. Crit. Rev. Biochem. Mol. Biol. **27**, 283-335
113 Oppermann, U. C., Filling, C. and Jornvall, H. (2001) Forms and functions of human SDR enzymes. Chem.-Biol. Interact. **130-132**, 699-705
114 Jarabak, J. (1992) Polycyclic aromatic hydrocarbon quinones may be either substrates for or irreversible inhibitors of the human placental NAD-linked 15-hydroxyprostaglandin dehydrogenase. Arch. Biochem. Biophys. **292**, 239-43
115 Maser, E. and Oppermann, U. C. (1997) Role of type-1 11β-hydroxysteroid dehydrogenase in detoxification processes. Eur. J. Biochem. **249**, 365-9
116 Maser, E., Wsol, V. and Martin, H. J. (2006) 11β-Hydroxysteroid dehydrogenase type 1: purification from human liver and characterization as carbonyl reductase of xenobiotics. Mol. Cell. Endocrinol. **248**, 34-7
117 Wsol, V., Szotakova, B., Skalova, L. and Maser, E. (2004) The novel anticancer drug oracin: different stereospecificity and cooperativity for carbonyl reduction by purified human liver 11β-hydroxysteroid dehydrogenase type 1. Toxicology **197**, 253-61
118 Yan, M., Rerko, R. M., Platzer, P., Dawson, D., Willis, J., Tong, M., Lawrence, E., Lutterbaugh, J., Lu, S., Willson, J. K., Luo, G., Hensold, J., Tai, H. H., Wilson, K. and Markowitz, S. D. (2004) 15-Hydroxyprostaglandin dehydrogenase, a COX-2 oncogene antagonist, is a TGF-β-induced suppressor of human gastrointestinal cancers. Proc. Natl. Acad. Sci. U. S. A. **101**, 17468-73

119 Änggård, E. and Samuelsson, B. (1965) Biosynthesis of prostaglandins from arachidonic acid in guinea pig lung. Prostaglandins and related factors. 38. J. Biol. Chem. **240**, 3518-21
120 Quinn, A. M., Harvey, R. G. and Penning, T. M. (2008) Oxidation of PAH trans-dihydrodiols by human aldo-keto reductase AKR1B10. Chem. Res. Toxicol., in press
121 Bianchet, M. A., Erdemli, S. B. and Amzel, L. M. (2008) Structure, function, and mechanism of cytosolic quinone reductases. Vitam. Horm. **78**, 63-84
122 Beyer, R. E., Segura-Aguilar, J., Di Bernardo, S., Cavazzoni, M., Fato, R., Fiorentini, D., Galli, M. C., Setti, M., Landi, L. and Lenaz, G. (1996) The role of DT-diaphorase in the maintenance of the reduced antioxidant form of coenzyme Q in membrane systems. Proc. Natl. Acad. Sci. U. S. A. **93**, 2528-32
123 Bianchet, M. A., Faig, M. and Amzel, L. M. (2004) Structure and mechanism of NAD[P]H:quinone acceptor oxidoreductases (NQO). Methods Enzymol. **382**, 144-74
124 Knox, R. J., Jenkins, T. C., Hobbs, S. M., Chen, S., Melton, R. G. and Burke, P. J. (2000) Bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) by human NAD(P)H quinone oxidoreductase 2: a novel co-substrate-mediated antitumor prodrug therapy. Cancer Res. **60**, 4179-86
125 Riley, R. J. and Workman, P. (1992) DT-diaphorase and cancer chemotherapy. Biochem. Pharmacol. **43**, 1657-69
126 Celli, C. M., Tran, N., Knox, R. and Jaiswal, A. K. (2006) NRH:quinone oxidoreductase 2 (NQO2) catalyzes metabolic activation of quinones and anti-tumor drugs. Biochem. Pharmacol. **72**, 366-76
127 Gong, X., Kole, L., Iskander, K. and Jaiswal, A. K. (2007) NRH:quinone oxidoreductase 2 and NAD(P)H:quinone oxidoreductase 1 protect tumor suppressor p53 against 20S proteasomal degradation leading to stabilization and activation of p53. Cancer Res. **67**, 5380-8
128 Garate, M., Wong, R. P., Campos, E. I., Wang, Y. and Li, G. (2008) NAD(P)H quinone oxidoreductase 1 inhibits the proteasomal degradation of the tumour suppressor p33(ING1b). EMBO Reports
129 Wermuth, B., Platts, K. L., Seidel, A. and Oesch, F. (1986) Carbonyl reductase provides the enzymatic basis of quinone detoxication in man. Biochem. Pharmacol. **35**, 1277-82
130 Schlegel, B. P., Ratnam, K. and Penning, T. M. (1998) Retention of NADPH-linked quinone reductase activity in an aldo-keto reductase following mutation of the catalytic tyrosine. Biochemistry **37**, 11003-11
131 O'Connor, T., Ireland, L. S., Harrison, D. J. and Hayes, J. D. (1999) Major differences exist in the function and tissue-specific expression of human aflatoxin B1 aldehyde reductase and the principal human aldo-keto reductase AKR1 family members. Biochem. J. **343 Pt 2**, 487-504
132 Forrest, G. L. and Gonzalez, B. (2000) Carbonyl reductase. Chem.-Biol. Interact. **129**, 21-40
133 Long, D. J., 2nd and Jaiswal, A. K. (2000) NRH:quinone oxidoreductase2 (NQO2). Chem.-Biol. Interact. **129**, 99-112
134 Ernster, L., Danielson, L. and Ljunggren, M. (1962) DT diaphorase. I. Purification from the soluble fraction of rat-liver cytoplasm, and properties. Biochim. Biophys. Acta **58**, 171-88
135 Ahmed, N. K., Felsted, R. L. and Bachur, N. R. (1979) Comparison and characterization of mammalian xenobiotic ketone reductases. J. Pharmacol. Exp. Ther. **209**, 12-9
136 Forman, H. J. and Dickinson, D. A. (2002) in Thiol Metabolism and Redox Regulation of Cellular Functions (Pompella, A., Bánhegyi, G. and Wellman-Rousseau, M., eds.), IOS Press, Amsterdam
137 Pastore, A., Federici, G., Bertini, E. and Piemonte, F. (2003) Analysis of glutathione: implication in redox and detoxification. Clin. Chim. Acta **333**, 19-39
138 Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R. and Turner, N. D. (2004) Glutathione metabolism and its implications for health. J. Nutr. **134**, 489-92

139 Habig, W. H. and Jakoby, W. B. (1981) Glutathione S-transferases (rat and human). Methods Enzymol. **77**, 218-31
140 Pearson, W. R. (2005) Phylogenies of glutathione transferase families. Methods Enzymol. **401**, 186-204
141 Eaton, D. L. and Bammler, T. K. (1999) Concise review of the glutathione S-transferases and their significance to toxicology. Toxicol. Sci. **49**, 156-64
142 Liu, S., Zhang, P., Ji, X., Johnson, W. W., Gilliland, G. L. and Armstrong, R. N. (1992) Contribution of tyrosine 6 to the catalytic mechanism of isoenzyme 3-3 of glutathione S-transferase. J. Biol. Chem. **267**, 4296-9
143 Sun, T. H. and Morgenstern, R. (1997) Binding of glutathione and an inhibitor to microsomal glutathione transferase. Biochem. J. **326 (Pt 1)**, 193-6
144 Dirr, H. W., Mann, K., Huber, R., Ladenstein, R. and Reinemer, P. (1991) Class π glutathione S-transferase from pig lung. Purification, biochemical characterization, primary structure and crystallization. Eur. J. Biochem. **196**, 693-8
145 Tellez-Sanz, R., Cesareo, E., Nuccetelli, M., Aguilera, A. M., Baron, C., Parker, L. J., Adams, J. J., Morton, C. J., Lo Bello, M., Parker, M. W. and Garcia-Fuentes, L. (2006) Calorimetric and structural studies of the nitric oxide carrier S-nitrosoglutathione bound to human glutathione transferase P1-1. Protein Sci. **15**, 1093-105
146 Vondracek, M., Xi, Z., Larsson, P., Baker, V., Mace, K., Pfeifer, A., Tjalve, H., Donato, M. T., Gomez-Lechon, M. J. and Grafström, R. C. (2001) Cytochrome P450 expression and related metabolism in human buccal mucosa. Carcinogenesis **22**, 481-8
147 Vondracek, M., Weaver, D. A., Sarang, Z., Hedberg, J. J., Willey, J. C., Wärngård, L. and Grafström, R. C. (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**, 776-82
148 Hedberg, J. J., Grafström, R. C., Vondracek, M., Sarang, Z., Wärngård, L. and Höög, J. O. (2001) Micro-array chip analysis of carbonyl-metabolising enzymes in normal, immortalised and malignant human oral keratinocytes. Cell. Mol. Life Sci. **58**, 1719-26
149 Hedberg, J. J., Hansson, A., Nilsson, J. A., Höög, J. O. and Grafström, R. C. (2001) Uniform expression of alcohol dehydrogenase 3 in epithelia regenerated with cultured normal, immortalised and malignant human oral keratinocytes. Altern. Lab. Anim. **29**, 325-33
150 Danielsson, O. and Jörnvall, H. (1992) "Enzymogenesis": classical liver alcohol dehydrogenase origin from the glutathione-dependent formaldehyde dehydrogenase line. Proc. Natl. Acad. Sci. U. S. A. **89**, 9247-51
151 Jörnvall, H., Höög, J. O. and Persson, B. (1999) SDR and MDR: completed genome sequences show these protein families to be large, of old origin, and of complex nature. FEBS Lett. **445**, 261-4
152 Jörnvall, H., Shafqat, J. and Persson, B. (2001) Variations and constant patterns in eukaryotic MDR enzymes. Conclusions from novel structures and characterized genomes. Chem.-Biol. Interact. **130-132**, 491-8
153 Nordling, E., Persson, B. and Jörnvall, H. (2002) Differential multiplicity of MDR alcohol dehydrogenases: enzyme genes in the human genome versus those in organisms initially studied. Cell. Mol. Life Sci. **59**, 1070-5
154 Norin, A., Shafqat, J., El-Ahmad, M., Alvelius, G., Cederlund, E., Hjelmqvist, L. and Jörnvall, H. (2004) Class III alcohol dehydrogenase: consistent pattern complemented with the mushroom enzyme. FEBS Lett. **559**, 27-32
155 Canestro, C., Albalat, R., Hjelmqvist, L., Godoy, L., Jornvall, H. and Gonzalez-Duarte, R. (2002) Ascidian and amphioxus Adh genes correlate functional and molecular features of the ADH family expansion during vertebrate evolution. J. Mol. Evol. **54**, 81-9
156 Molotkov, A., Fan, X., Deltour, L., Foglio, M. H., Martras, S., Farrés, J., Parés, X. and Duester, G. (2002) Stimulation of retinoic acid production and growth by ubiquitously expressed alcohol dehydrogenase Adh3. Proc. Natl. Acad. Sci. U. S. A. **99**, 5337-42

157 Lee, S. L., Wang, M. F., Lee, A. I. and Yin, S. J. (2003) The metabolic role of human ADH3 functioning as ethanol dehydrogenase. FEBS Lett. **544**, 143-7
158 Moulis, J. M., Holmquist, B. and Vallee, B. L. (1991) Hydrophobic anion activation of human liver χχ alcohol dehydrogenase. Biochemistry **30**, 5743-9
159 Jörnvall, H. and Höög, J. O. (1995) Nomenclature of alcohol dehydrogenases. Alcohol Alcohol. **30**, 153-61
160 Duester, G., Farrés, J., Felder, M. R., Holmes, R. S., Höög, J. O., Parés, X., Plapp, B. V., Yin, S. J. and Jörnvall, H. (1999) Recommended nomenclature for the vertebrate alcohol dehydrogenase gene family. Biochem. Pharmacol. **58**, 389-95
161 Wu, H., Romieu, I., Sienra-Monge, J. J., Estela Del Rio-Navarro, B., Anderson, D. M., Jenchura, C. A., Li, H., Ramirez-Aguilar, M., Del Carmen Lara-Sanchez, I. and London, S. J. (2007) Genetic variation in S-nitrosoglutathione reductase (GSNOR) and childhood asthma. J. Allergy Clin. Immunol. **120**, 322-8
162 Yang, Z. N., Bosron, W. F. and Hurley, T. D. (1997) Structure of human χχ alcohol dehydrogenase: a glutathione-dependent formaldehyde dehydrogenase. J. Mol. Biol. **265**, 330-43
163 Sanghani, P. C., Robinson, H., Bennett-Lovsey, R., Hurley, T. D. and Bosron, W. F. (2003) Structure-function relationships in human Class III alcohol dehydrogenase (formaldehyde dehydrogenase). Chem.-Biol. Interact. **143-144**, 195-200
164 Engeland, K., Höög, J. O., Holmquist, B., Estonius, M., Jörnvall, H. and Vallee, B. L. (1993) Mutation of Arg-115 of human class III alcohol dehydrogenase: a binding site required for formaldehyde dehydrogenase activity and fatty acid activation. Proc. Natl. Acad. Sci. U. S. A. **90**, 2491-4
165 Sanghani, P. C., Bosron, W. F. and Hurley, T. D. (2002) Human glutathione-dependent formaldehyde dehydrogenase. Structural changes associated with ternary complex formation. Biochemistry **41**, 15189-94
166 Sanghani, P. C., Robinson, H., Bosron, W. F. and Hurley, T. D. (2002) Human glutathione-dependent formaldehyde dehydrogenase. Structures of apo, binary, and inhibitory ternary complexes. Biochemistry **41**, 10778-86
167 Sanghani, P. C., Stone, C. L., Ray, B. D., Pindel, E. V., Hurley, T. D. and Bosron, W. F. (2000) Kinetic mechanism of human glutathione-dependent formaldehyde dehydrogenase. Biochemistry **39**, 10720-9
168 Feldman, M. Y. (1973) Reactions of nucleic acids and nucleoproteins with formaldehyde. Prog. Nucleic Acid Res. Mol. Biol. **13**, 1-49
169 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
170 Leikauf, G. D. (2002) Hazardous air pollutants and asthma. Environ. Health Perspect. **110 Suppl 4**, 505-26
171 Dales, R. and Raizenne, M. (2004) Residential exposure to volatile organic compounds and asthma. J. Asthma **41**, 259-70
172 Casset, A., Marchand, C., Purohit, A., le Calve, S., Uring-Lambert, B., Donnay, C., Meyer, P. and de Blay, F. (2006) Inhaled formaldehyde exposure: effect on bronchial response to mite allergen in sensitized asthma patients. Allergy **61**, 1344-50
173 Krzyzanowski, M., Quackenboss, J. J. and Lebowitz, M. D. (1990) Chronic respiratory effects of indoor formaldehyde exposure. Environ. Res. **52**, 117-25
174 Neuberger, A. (1981) in Compr. Biochem., vol. 19A (Neuberger, A. and van Deenen, L. L. M., eds.), pp. 257–303, Elsevier, Amsterdam
175 Kucharczyk, N., Yang, J. T., Wong, K. K. and Sofia, R. D. (1984) The formaldehyde-donating activity of N^5,N^{10}-methylene tetrahydrofolic acid in xenobiotic biotransformation. Xenobiotica **14**, 667-76
176 Lutz, W. K. (1990) Endogenous genotoxic agents and processes as a basis of spontaneous carcinogenesis. Mutat. Res. **238**, 287-95
177 Smith, T. J., Guo, Z., Gonzalez, F. J., Guengerich, F. P., Stoner, G. D. and Yang, C. S. (1992) Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-

butanone in human lung and liver microsomes and cytochromes P-450 expressed in hepatoma cells. Cancer Res. **52**, 1757-63
178 Waydhas, C., Weigl, K. and Sies, H. (1978) The disposition of formaldehyde and formate arising from drug N-demethylations dependent on cytochrome P-450 in hepatocytes and in perfused rat liver. Eur. J. Biochem. **89**, 143-50
179 Cinti, D. L., Keyes, S. R., Lemelin, M. A., Denk, H. and Schenkman, J. B. (1976) Biochemical properties of rat liver mitochondrial aldehyde dehydrogenase with respect to oxidation of formaldehyde. J. Biol. Chem. **251**, 1571-7
180 Siew, C., Deitrich, R. A. and Erwin, V. G. (1976) Localization and characteristics of rat liver mitochondrial aldehyde dehydrogenases. Arch. Biochem. Biophys. **176**, 638-49
181 Mukerjee, N. and Pietruszko, R. (1992) Human mitochondrial aldehyde dehydrogenase substrate specificity: comparison of esterase with dehydrogenase reaction. Arch. Biochem. Biophys. **299**, 23-9
182 Williamson, D. H., Lund, P. and Krebs, H. A. (1967) The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. Biochem. J. **103**, 514-27
183 Kümmerle, N., Feucht, H. H. and Kaulfers, P. M. (1996) Plasmid-mediated formaldehyde resistance in Escherichia coli: characterization of resistance gene. Antimicrob. Agents Chemother. **40**, 2276-9
184 Dorsey, C. W. and Actis, L. A. (2004) Analysis of pVU3695, a plasmid encoding glutathione-dependent formaldehyde dehydrogenase activity and formaldehyde resistance in the Escherichia coli VU3695 clinical strain. Plasmid **51**, 116-26
185 Achkor, H., Diaz, M., Fernandez, M. R., Biosca, J. A., Pares, X. and Martinez, M. C. (2003) Enhanced formaldehyde detoxification by overexpression of glutathione-dependent formaldehyde dehydrogenase from Arabidopsis. Plant Physiol. **132**, 2248-55
186 Hess, D. T., Matsumoto, A., Kim, S. O., Marshall, H. E. and Stamler, J. S. (2005) Protein S-nitrosylation: purview and parameters. Nat. Rev. Mol. Cell Biol. **6**, 150-66
187 Mannick, J. B. and Schonhoff, C. M. (2004) NO means no and yes: regulation of cell signaling by protein nitrosylation. Free Radic. Res. **38**, 1-7
188 Gaston, B., Reilly, J., Drazen, J. M., Fackler, J., Ramdev, P., Arnelle, D., Mullins, M. E., Sugarbaker, D. J., Chee, C., Singel, D. J., Loscalzo, J. and Stamler, J. S. (1993) Endogenous nitrogen oxides and bronchodilator S-nitrosothiols in human airways. Proc. Natl. Acad. Sci. U. S. A. **90**, 10957-61
189 Hou, Y., Guo, Z., Li, J. and Wang, P. G. (1996) Seleno compounds and glutathione peroxidase catalyzed decomposition of S-nitrosothiols. Biochem. Biophys. Res. Commun. **228**, 88-93
190 Nikitovic, D. and Holmgren, A. (1996) S-nitrosoglutathione is cleaved by the thioredoxin system with liberation of glutathione and redox regulating nitric oxide. J. Biol. Chem. **271**, 19180-5
191 Trujillo, M., Alvarez, M. N., Peluffo, G., Freeman, B. A. and Radi, R. (1998) Xanthine oxidase-mediated decomposition of S-nitrosothiols. J. Biol. Chem. **273**, 7828-34
192 Jourd'heuil, D., Laroux, F. S., Miles, A. M., Wink, D. A. and Grisham, M. B. (1999) Effect of superoxide dismutase on the stability of S-nitrosothiols. Arch. Biochem. Biophys. **361**, 323-30
193 Khan, M., Sekhon, B., Giri, S., Jatana, M., Gilg, A. G., Ayasolla, K., Elango, C., Singh, A. K. and Singh, I. (2005) S-Nitrosoglutathione reduces inflammation and protects brain against focal cerebral ischemia in a rat model of experimental stroke. J. Cereb. Blood Flow Metab. **25**, 177-92
194 Ju, T. C., Chen, S. D., Liu, C. C. and Yang, D. I. (2005) Protective effects of S-nitrosoglutathione against amyloid β-peptide neurotoxicity. Free Radic. Biol. Med. **38**, 938-49
195 Schonhoff, C. M., Matsuoka, M., Tummala, H., Johnson, M. A., Estevez, A. G., Wu, R., Kamaid, A., Ricart, K. C., Hashimoto, Y., Gaston, B., Macdonald, T.

L., Xu, Z. and Mannick, J. B. (2006) S-nitrosothiol depletion in amyotrophic lateral sclerosis. Proc. Natl. Acad. Sci. U. S. A. **103**, 2404-9
196 Prasad, R., Giri, S., Nath, N., Singh, I. and Singh, A. K. (2007) GSNO attenuates EAE disease by S-nitrosylation-mediated modulation of endothelial-monocyte interactions. Glia **55**, 65-77
197 Gaston, B., Sears, S., Woods, J., Hunt, J., Ponaman, M., McMahon, T. and Stamler, J. S. (1998) Bronchodilator S-nitrosothiol deficiency in asthmatic respiratory failure. Lancet **351**, 1317-9
198 Hart, T. W. (1985) Some observations concerning the S-nitroso and S-phenylsulphonyl derivatives of L-cysteine and glutathione. Tetrahedron Lett. **26**, 2013-6
199 Abagyan, R. and Totrov, M. (1994) Biased probability Monte Carlo conformational searches and electrostatic calculations for peptides and proteins. J. Mol. Biol. **235**, 983-1002
200 Abagyan, R. A., Totrov, M. M. and Kuznetsov, D. N. (1994) ICM - a new method for protein modeling and design. Applications to docking and structure prediction from the distorted native conformation. J. Comput. Chem. **15**, 488-506
201 Nemethy, G., Gibson, K. D., Palmer, K. A., Yoon, C. N., Paterlini, G., Zagari, A., Rumsey, S. and Scheraga, H. A. (1992) Energy parameters in polypeptides. 10. Improved geometrical parameters and nonbonded interactions for use in the ECEPP/3 algorithm, with application to proline-containing peptides. J. Phys. Chem. **96**, 6472-6484
202 Arnelle, D. R. and Stamler, J. S. (1995) NO^+, NO, and NO^- donation by S-nitrosothiols: implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation. Arch. Biochem. Biophys. **318**, 279-85
203 Chu, P., Wu, E. and Weiss, L. M. (2000) Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases. Mod. Pathol. **13**, 962-72
204 Vaidya, M. M., Borges, A. M., Pradhan, S. A., Rajpal, R. M. and Bhisey, A. N. (1989) Altered keratin expression in buccal mucosal squamous cell carcinoma. J. Oral Pathol. Med. **18**, 282-6
205 Leonardi, R., Pannone, G., Magro, G., Kudo, Y., Takata, T. and Lo Muzio, L. (2002) Differential expression of heat shock protein 27 in normal oral mucosa, oral epithelial dysplasia and squamous cell carcinoma. Oncol. Rep. **9**, 261-6
206 Fan, G. K., Chen, J., Ping, F. and Geng, Y. (2006) Immunohistochemical analysis of P57(kip2), p53 and hsp60 expressions in premalignant and malignant oral tissues. Oral Oncol. **42**, 147-53
207 Kaur, J., Srivastava, A. and Ralhan, R. (1998) Expression of 70-kDa heat shock protein in oral lesions: marker of biological stress or pathogenicity. Oral Oncol. **34**, 496-501
208 Goto, Y., Sueoka, E., Chiba, H. and Fujiki, H. (1999) Significance of heterogeneous nuclear ribonucleoprotein B1 as a new early detection marker for oral squamous cell carcinoma. Jpn. J. Cancer Res. **90**, 1358-63
209 Gasco, M., Bell, A. K., Heath, V., Sullivan, A., Smith, P., Hiller, L., Yulug, I., Numico, G., Merlano, M., Farrell, P. J., Tavassoli, M., Gusterson, B. and Crook, T. (2002) Epigenetic inactivation of 14-3-3 σ in oral carcinoma: association with p16(INK4a) silencing and human papillomavirus negativity. Cancer Res. **62**, 2072-6
210 Chen, J., He, Q. Y., Yuen, A. P. and Chiu, J. F. (2004) Proteomics of buccal squamous cell carcinoma: the involvement of multiple pathways in tumorigenesis. Proteomics **4**, 2465-75
211 Taylor, W. R. and Stark, G. R. (2001) Regulation of the G2/M transition by p53. Oncogene **20**, 1803-15
212 Mosser, D. D., Duchaine, J. and Massie, B. (1993) The DNA-binding activity of the human heat shock transcription factor is regulated in vivo by hsp70. Mol. Cell. Biol. **13**, 5427-38
213 Tian, Q., Stepaniants, S. B., Mao, M., Weng, L., Feetham, M. C., Doyle, M. J., Yi, E. C., Dai, H., Thorsson, V., Eng, J., Goodlett, D., Berger, J. P., Gunter, B.,

Linseley, P. S., Stoughton, R. B., Aebersold, R., Collins, S. J., Hanlon, W. A. and Hood, L. E. (2004) Integrated genomic and proteomic analyses of gene expression in Mammalian cells. Mol. Cell. Proteomics **3**, 960-9

214 Greenbaum, D., Jansen, R. and Gerstein, M. (2002) Analysis of mRNA expression and protein abundance data: an approach for the comparison of the enrichment of features in the cellular population of proteins and transcripts. Bioinformatics **18**, 585-96

215 Saranath, D., Panchal, R. G., Nair, R., Mehta, A. R., Sanghavi, V., Sumegi, J., Klein, G. and Deo, M. G. (1989) Oncogene amplification in squamous cell carcinoma of the oral cavity. Jpn. J. Cancer Res. **80**, 430-7

216 Field, J. K. (1995) The role of oncogenes and tumour-suppressor genes in the aetiology of oral, head and neck squamous cell carcinoma. J. R. Soc. Med. **88**, 35P-39P

217 Ohkura, S., Kondoh, N., Hada, A., Arai, M., Yamazaki, Y., Sindoh, M., Takahashi, M., Matsumoto, I. and Yamamoto, M. (2005) Complex formations involving both SP-1 and SP-3 at the transcriptional regulatory sequence correlate with the activation of the Keratin 14 gene in human oral squamous cell carcinoma cells. Oncol. Rep. **14**, 1577-81

218 Chen, Y. K., Huse, S. S. and Lin, L. M. (2003) Differential expression of p53, p63 and p73 proteins in human buccal squamous-cell carcinomas. Clin. Otolaryngol. Allied Sci. **28**, 451-5

219 Iamaroon, A., Pattamapun, K. and Piboonniyom, S. O. (2006) Aberrant expression of Smad4, a TGF-beta signaling molecule, in oral squamous cell carcinoma. J. Oral Sci. **48**, 105-9

220 Bindhu, O. S., Ramadas, K., Sebastian, P. and Pillai, M. R. (2006) High expression levels of nuclear factor κ B and gelatinases in the tumorigenesis of oral squamous cell carcinoma. Head Neck **28**, 916-25

221 Harris, G., Ghazallah, R. A., Nascene, D., Wuertz, B. and Ondrey, F. G. (2005) PPAR activation and decreased proliferation in oral carcinoma cells with 4-HPR. Otolaryngol. Head Neck Surg. **133**, 695-701

222 Brennan, P. A., Mackenzie, N. and Quintero, M. (2005) Hypoxia-inducible factor 1α in oral cancer. J. Oral Pathol. Med. **34**, 385-9

223 Krauss, G. (1997) Biochemie der Regulation und Signaltransduktion, Wiley-VCH, Weinheim

224 Tusher, V. G., Tibshirani, R. and Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. U. S. A. **98**, 5116-21

225 Yoon, K. A., Nakamura, Y. and Arakawa, H. (2004) Identification of ALDH4 as a p53-inducible gene and its protective role in cellular stresses. J. Hum. Genet. **49**, 134-40

226 Truong, A. B. and Khavari, P. A. (2007) Control of keratinocyte proliferation and differentiation by p63. Cell Cycle **6**, 295-9

227 Tong, M., Ding, Y. and Tai, H. H. (2006) Histone deacetylase inhibitors and transforming growth factor-β induce 15-hydroxyprostaglandin dehydrogenase expression in human lung adenocarcinoma cells. Biochem. Pharmacol. **72**, 701-9

228 Masui, T., Wakefield, L. M., Lechner, J. F., LaVeck, M. A., Sporn, M. B. and Harris, C. C. (1986) Type β transforming growth factor is the primary differentiation-inducing serum factor for normal human bronchial epithelial cells. Proc. Natl. Acad. Sci. U. S. A. **83**, 2438-42

229 Tai, H. H., Tong, M. and Ding, Y. (2007) 15-hydroxyprostaglandin dehydrogenase (15-PGDH) and lung cancer. Prostaglandins Other Lipid Mediat. **83**, 203-8

230 Barski, O. A., Papusha, V. Z., Kunkel, G. R. and Gabbay, K. H. (2004) Regulation of aldehyde reductase expression by STAF and CHOP. Genomics **83**, 119-29

231 Maytin, E. V. and Habener, J. F. (1998) Transcription factors C/EBP α, C/EBP β, and CHOP (Gadd153) expressed during the differentiation program of keratinocytes in vitro and in vivo. J. Invest. Dermatol. **110**, 238-46

232 Forte-McRobbie, C. M. and Pietruszko, R. (1986) Purification and characterization of human liver "high K_m" aldehyde dehydrogenase and its identification as glutamic γ-semialdehyde dehydrogenase. J. Biol. Chem. **261**, 2154-63
233 Komoto, J., Yamada, T., Watanabe, K. and Takusagawa, F. (2004) Crystal structure of human prostaglandin F synthase (AKR1C3). Biochemistry **43**, 2188-98
234 Sato, S. and Kador, P. F. (1990) Inhibition of aldehyde reductase by aldose reductase inhibitors. Biochem. Pharmacol. **40**, 1033-42
235 Sanghani, P. C., Davis, W. I., Zhai, L. and Robinson, H. (2006) Structure-function relationships in human glutathione-dependent formaldehyde dehydrogenase. Role of Glu-67 and Arg-368 in the catalytic mechanism. Biochemistry **45**, 4819-30
236 Fernandez, M. R., Biosca, J. A. and Parés, X. (2003) S-nitrosoglutathione reductase activity of human and yeast glutathione-dependent formaldehyde dehydrogenase and its nuclear and cytoplasmic localisation. Cell. Mol. Life Sci. **60**, 1013-8
237 Thompson, C. M. and Grafström, R. C. (2008) Mechanistic considerations for formaldehyde-induced bronchoconstriction involving S-nitrosoglutathione reductase. J. Toxicol. Environ. Health **71**, 244-8
238 Söderdahl, T., Enoksson, M., Lundberg, M., Holmgren, A., Ottersen, O. P., Orrenius, S., Bolcsfoldi, G. and Cotgreave, I. A. (2003) Visualization of the compartmentalization of glutathione and protein-glutathione mixed disulfides in cultured cells. FASEB J. **17**, 124-6
239 Townsend, D. M., Tew, K. D. and Tapiero, H. (2003) The importance of glutathione in human disease. Biomed. Pharmacother. **57**, 145-55
240 Hedberg, J. J., Strömberg, P. and Höög, J. O. (1998) An attempt to transform class characteristics within the alcohol dehydrogenase family. FEBS Lett. **436**, 67-70
241 Cho, H. and Plapp, B. V. (1998) Specificity of alcohol dehydrogenases for sulfoxides. Biochemistry **37**, 4482-9