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Use of Human *In Vitro* Skin Models for Accurate and Ethical Risk Assessment: Metabolic Considerations

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Several human skin models employing primary cells and immortalized cell lines used as monocultures or combined to produce reconstituted 3D skin constructs have been developed. Furthermore, these models have been included in European genotoxicity and sensitization/irritation assay validation projects. In order to help interpret data, Cosmetics Europe (formerly COLIPA) facilitated research projects that measured a variety of defined phase I and II enzyme activities and created a complete proteomic profile of xenobiotic metabolizing enzymes (XMEs) in native human skin and compared them with data obtained from a number of *in vitro* models of human skin. Here, we have summarized our findings on the current knowledge of the metabolic capacity of native human skin and *in vitro* models and made an overall assessment of the metabolic capacity from gene expression, proteomic expression, and substrate metabolism data. The known low expression and function of phase I enzymes in native whole skin were reflected in the *in vitro* models. Some XMEs in whole skin were not detected in *in vitro* models and vice versa, and some major hepatic XMEs such as cytochrome P450-monooxygenases were absent or measured only at very low levels in the skin. Conversely, despite varying mRNA and protein levels of phase II enzymes, functional activity of glutathione S-transferases, N-acetyltransferase 1, and UDP-glucuronosyltransferases were all readily measurable in whole skin and *in vitro* skin models at activity levels similar to those measured in the liver. These projects have enabled a better understanding of the contribution of XMEs to toxicity endpoints.

Key Words: **human 3D skin models; keratinocytes; phase I and II metabolism; proteomics; Cosmetics Europe.**

Several human skin models employing various primary cells and immortalized cell lines, used as monocultures or combined to produce reconstituted 3D skin constructs, have been developed. Because *in vivo* models for skin irritation and genotoxicity have a number of disadvantages, such as species-specific metabolism, the development of human skin models represents an opportunity to use more predictive and relevant (human specific), as well as ethically acceptable, assays for human exposure and risk assessment. The emphasis on developing *in vitro* skin models to replace the *in vivo* equivalents has been increased further by the Seventh Amendment of the Cosmetics Directive (EU, 2003) and the REACh regulations (European Commission, 2006). As a result, alternative skin models have been included in European genotoxicity and sensitization/irritation assay validation projects. Until now, little has been published on the capacity of the different skin models used to metabolize xenobiotics. One of the methods by which researchers have characterized metabolic profiles of human skin is mRNA expression analysis. Although the presence of mRNA, which encodes for a specific enzyme, is an indication that the protein could be synthesized, this does not always correlate with the presence of the respective protein or indeed with its function (Pushparajah *et al.*, 2008; Rodríguez-Antona *et al.*, 2002), leaving some question over the relevance of such measurements. For this reason, Cosmetics Europe (formerly COLIPA: www.cosmeticseurope.eu) initiated two collaborative projects to investigate xenobiotic metabolism in human skin models more fully.

One Cosmetics Europe project focused on the protein expression of xenobiotic metabolizing enzymes (XMEs) in *ex vivo* whole human skin, 3D models (EpiDerm and SkinEthic reconstructed human epidermis [RHE] models), and monolayer cultures of HaCaT cells. To this end, a label-free proteomic technique involving liquid chromatography tandem mass spectrometry analysis of tryptic peptides derived from proteins present was used. The technique was shown to have a limit of detection

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(LOD) of 0.1–0.2 pmol/mg microsomal protein for CYP1A1, CYP2E1, CYP3A4, and CYP3A5, making it more sensitive than traditional immunoblotting that detects down to ~2.5 pmol/mg microsomal protein (Westlind-Johnsson *et al.*, 2003). In this technique, the more tryptic peptides detected, the greater the certainty of the identification, and, in general, the greater is the abundance of the protein. In this study, all of the proteins considered were identified from a minimum of two unique tryptic peptides. This technique allowed for a comprehensive analysis of many proteins (> 2000) in human native skin including 36 XMEs. Such a comprehensive analysis would not have been possible using enzyme activity assays or by immunoblotting as there are relatively few assays specific to individual XMEs that have been established, and there is a lack of suitable antibodies. The results of this project were published by van Eijl *et al.* (2012).

A second Cosmetics Europe project involved measuring phase I (Götz *et al.*, 2012a) and phase II (Götz *et al.*, 2012b) XME activities in *ex vivo* human skin, EpiDerm model, keratinocyte-based cell lines, HaCaT (Boukamp *et al.*, 1988) and NCTC 2544 (Bakken *et al.*, 1961), and primary normal human epidermal keratinocytes (NHEKs). Although some phase I activities could be detected using substrates that are metabolized at relatively low rates (by more than one CYP enzyme) to fluorescent metabolites, others were too low to be detected and should be studied using substrates with a higher turnover and more sensitive techniques, such as liquid chromatography tandem mass spectrometry. This project investigated the basal levels and, in some cases, induced levels of a number of XMEs using prototypical substrates as markers for activities. It was shown that the sensitivity of the assays could be increased by increasing the duration of the incubation; therefore, the effect of test compounds on the enzyme activities in the EpiDerm model could be incorporated into the typical micronucleus assay using this model—the so-called reconstructed skin micronucleus (RSMN) assay (Götz *et al.*, 2012c).

Here, we review the metabolic capacity of 2D and 3D *in vitro* skin models based on the two Cosmetics Europe projects and information gained from the literature—mainly from Hu *et al.* (2010) and Luu-The *et al.* (2009). Initially, there was a focus on the metabolic activity of *in vitro* models used for the measurement of sensitization, which included HaCaT and NCTC 2544 cells. Subsequently, the role of metabolism in genotoxicity assays using 3D skin models was also included. For this reason, the metabolism project encompassed both toxicity endpoints. Overviews of some of the most notable differences/similarities between whole skin and the *in vitro* 2D and 3D models are shown in Tables 1 and 2. We also relate the outcome of the results with respect to the assays in which each model is used for risk assessment.

2D Culture Models

There are numerous sources of adult human keratinocytes; however, they may vary considerably with respect to their metabolic capacity due to the conditions in which they are cultured (Berghard *et al.*, 1990). In order to try to standardize and maintain a consistent phenotype of the cells, a number of established lines of human keratinocytes have been developed. These include HaCaT and NCTC 2544 cells, which were both studied in the Cosmetics Europe metabolic activity (but not proteomic) project and compared with NHEKs.

NCTC 2544 cells have been investigated as part of the EU project, SENS-IT-IV, as a promising *in vitro* model to identify *in vitro* contact allergens (Galbiati *et al.*, 2011). In addition, these cells have been used as a model to investigate mechanisms of phototoxicity (Viola *et al.*, 2007) and inflammation (Parodi *et al.*, 2010). XME activities (ethoxyresorufin *o*-deethylation [EROD] and pentoxyresorufin *o*-depentylation [PROD]) and their induction have been investigated by others and were detectable in these cells (Gelardi *et al.*, 2001). Likewise, in the Cosmetics Europe project, basal EROD activities were measurable in NCTC cells and were just above the LOD of resorufin produced but were induced significantly by the AhR agonist, 3-methylcholanthrene (Götz *et al.*, 2012a) (Table 1). The effects of enzyme inducers were measured to determine whether different models were responsive to induction and to demonstrate if the metabolic capacity of the models could be altered during the course of treatment with a test compound. Time-dependent induction of PROD by phenobarbital and inhibition by metyrapone of the induced levels were demonstrated by Gelardi *et al.* (2001), suggesting that these cells expressed functional CYP2B under the conditions used by these investigators. By contrast, PROD activity (basal or cells treated with the hepatic CYP2B6 inducers, rifampicin or 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-*o*-(3,4-dichlorobenzyl)oxime) and CYP2B6 protein were not detected in any of the *in vitro* models tested in the Cosmetics Europe project.

More extensive investigations were carried out with HaCaT cells in the Cosmetics Europe metabolism project in which enzyme activities and the proteomic XME profile were investigated. These have been used to identify skin sensitizers and predict local lymph node assay EC3 values by determining the expression of genes controlled by the antioxidant response element (e.g., NADH quinone oxidoreductase, interleukin-8, and aldoketo reductase [AKR] [www.ceetox.com]). The majority of chemical sensitizers can be inherently reactive, or "prehaptens" which form haptens by nonenzymatic processes, or they can be bioactivated to reactive metabolites. The latter bioactivation has been indirectly linked to dermal CYP1A, using incubations of a prohapten with a "skin-like P450 cocktail" (Hagvall *et al.*, 2008; Ott *et al.*, 2009). In addition, CYP1A1 and CYP1B1 are the main extrahepatic CYPs involved in the activation of chemical carcinogens (Gonzalez and Gelboin, 1994). Therefore, it was of interest to determine the activity of CYP1-mediated metabolism of appropriate substrates, namely 7-ethoxyresorufin, which is metabolized by CYP1A1, CYP1A2, and CYP1B1 (Shimada *et al.*, 1997), and methoxyresorufin, which is reported to be metabolized by CYP1A2

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TABLE 1
Comparative Evidence for Phase I and II XMEs from Protein and Activity Measurements in 2D Skin Models and Native Whole (i.e., Epidermis and Dermis) Human Skin

	Native skin		HaCaT		NCTC	
Enzyme	Protein	Activity	Protein	Activity	Protein	Activity
Phase I enzymes						
CYP1	X	✓ Low (at LOD)	X	✓ Low but inducible (at LOD)	ND	✓ Low but inducible (at LOD)
CYP2B6	X	X	X	X	ND	X
CYP3A	X	✓ (0.076 ± 41)	X	✓ (0.038 ± 13)	ND	✓ (0.033 ± 5)
COX-2	X	✓ (0.0235 ± 8.7)	X	✓ (0.00005 ± 0.03)	ND	✓ (0.00002 ± 0.01)
Catalase	✓	ND	✓	ND	ND	ND
Alcohol dehydrogenase 3	✓	ND	✓	ND	ND	ND
Alcohol dehydrogenase 1B (dermis)	✓	ND	X	ND	ND	ND
Epoxide hydrolase 1 (dermis)	✓	ND	X	ND	ND	ND
Amine oxidase (dermis)	✓	ND	X	ND	ND	ND
Aldehyde dehydrogenases (1L1, 3A2)	✓	ND	X	ND	ND	ND
AKR 1B	X	ND	✓	ND	ND	ND
Phase II enzymes						
COMT	X	ND	✓	ND	ND	
Glutathione S-transferase Pi, omega	✓	✓	✓	✓	ND	✓
Glutathione S-transferase alpha	✓	(20 ± 6.8)	X	(~50)	ND	(~50)
Glutathione S-transferase theta	✓		X		ND	
NAT10	X	✓	✓	✓	ND	✓
NAT1	X	(Slow = 0.63–0.94) (Fast = 1.73–3.03)	X	(0.65 ± 0.37)	ND	(0.35 ± 0.22)
Sulfotransferase 2B1	X	ND	X	ND	ND	ND
UGT	X	✓ (1.3 ± 0.2)	X	✓ (2.0 ± 1.0)	ND	✓ (0.9 ± 0.2)

Note. Other enzymes were detected but are not listed here. X, not detected; ✓, detected; ND, not determined. When measured, activities are shown within parentheses, and all are expressed as nmol product/min/mg protein. Values shown are mean ± SD, with the exception of NAT activity, which is given as a range of activities for slow and fast acetylators due to the polymorphism in this enzyme. Activities were measured as follows: CYP1 activity, EROD activity; *o*-deethylation of ethoxyresorufin (LOD was 0.0002 nmol product/min/mg protein); CYP3A activity, dealkylation of 7-benzyloxyquinoline; COX-2 activity, PGE_2 formation; glutathione S-transferase activity, GSH conjugation of the broad-spectrum substrate, CDNB; NAT, N-acetylation of the NAT1 and NAT2 substrates, *p*-toluidine; and UGT activity, depletion of the substrate, 4-methylumbelliferone.

(Burke *et al.*, 1994; Donato *et al.*, 2004). Gene expression of the CYP1 family was not included in the Cosmetics Europe studies, but the expression of these genes in HaCaT cells has been published by Delescluse *et al.* (1997). In the Cosmetics Europe studies, proteins belonging to the CYP families 1–3 were absent in HaCaT cells (Table 1). However, determination of EROD and methoxyresorufin *o*-demethylase (MROD) activities suggested that basal CYP1A/1B1 activities were measurable but only just above the LODs of the methods (EROD was 0.4 pmol/min/mg protein and was the same as in NHEKs) and that they were readily induced by 3-methylcholanthrene. The lack of correlation between the levels of protein and mRNA expression and activity can be due to the general low abundance of skin CYPs (the linear correlation can only occur with highly abundant CYPs), which was only measurable by the very sensitive mRNA analysis or by extending the incubation duration to 6 h. Ultimately, the turnover of a substrate is the true reflection of the metabolic capacity of a model and as EROD and MROD were detectable and inducible in HaCaT cells; this model was considered suitable for the testing of chemicals that require bioactivation by CYP1A enzymes and that could also induce these AhR-controlled enzymes.

Another phase I enzyme involved in the bioactivation of chemicals is cyclo-oxygenase-2 (COX-2) (Vogel, 2000). COX-2 is strongly inducible by growth factors, cytokines, and other stimuli such as UV radiation and is responsible for the production of prostaglandins (PGs) during inflammatory response (Fernau *et al.*, 2010). HaCaT cells were shown to produce PGE_2, but this COX-2-mediated metabolic activity was 100-fold lower in NHEKs, supporting the hypothesis that COX-2 activity was reduced as a result of immortalization (Zhao *et al.*, 2008). Measurable protein levels of aldehyde and alcohol dehydrogenases (not all listed in Table 1), as well as reductase and catalase, were detected in HaCaT cells, many of which were also present in native whole skin (the activities of which were not measured). Some enzymes present in whole skin,

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TABLE 2

Comparative Evidence for Phase I and II Enzyme XME Expression from mRNA, Protein and Activity Measurements in 3D Skin Models and Native Whole (i.e., Epidermis and Dermis) Human Skin

	Native skin			EpiDerm			EpiSkin		RHE
Enzyme	mRNA	Protein	Activity	mRNA	Protein	Activity	mRNA	Protein	Protein
Phase I enzymes									
CYP1	✓ Low	X	X	✓ Low	X	X	✓ Low	X	X
CYP2B6	✓ Low	X	X	X	X	X	✓ Low	X	X
CYP3A	✓ Low	X	✓	✓ Low	X	✓	✓ Low	X	X
FMO3 (Dermis)	✓ Low	X	ND	X	X	ND	✓/X Low/ Absent	X	X
FMO5	✓ Low	X	ND	✓	X	ND	✓/X Low/ Absent	X	X
COX-2 (mainly dermis)	✓	X	✓	NR	X	✓	✓ Low	X	ND
Catalase	NR	✓	ND	ND	✓	ND	NR	✓	✓
Alcohol dehydrogenase 3	NR	✓	ND	NR	✓	ND	NR	✓	✓
Aldehyde dehydrogenase 2	✓	✓	ND	✓	✓	ND	NR	✓	✓
Alcohol dehydrogenase 1B (dermis)	✓	✓	ND	✓	X	ND	✓	X	X
Epoxide hydrolase 1 (dermis)	✓	✓	ND	✓	X	ND	✓	X	X
Amine oxidase (dermis)	NR	✓	ND	NR	X	ND	NR	X	X
Alcohol dehydrogenase 4	NR	✓	ND	NR	X	ND	NR	X	X
Aldehyde dehydrogenases 1L1	NR	✓	ND	NR	X	ND	NR	X	X
Aldehyde dehydrogenases 3A2	NR	✓	ND	NR	X	ND	NR	✓	X
AKR 1B	NR	X	ND	NR	✓	ND	NR	✓	✓
Aldehyde dehydrogenase 7A1	✓	X	ND	✓	✓	ND	NR	X	✓
Phase II enzymes									
Glutathione S-transferase Pi, omega	✓	✓	✓	✓	✓	✓	✓	✓	✓
Glutathione S-transferase alpha	✓	✓		✓	✓		NR	✓	✓
Glutathione S-transferase theta	✓	✓		NR	X		✓	X	X
NAT10	NR	X	✓	NR	X	✓	NR	✓	X
NAT1	✓ Low	X		✓	X		✓ Low	X	X
Sulfotransferase 2B1	✓	X	ND	✓	✓	ND	✓	✓	✓
COMT	✓	X	ND	✓	✓	ND	✓	✓	✓
UGTs 1A	✓ Low	X	✓	✓	X	✓	✓	X	X
UGTs 2B	X	X		X	X		✓	X	X

Note. Other enzymes were detected but are not listed here. mRNA expression is as reported by Hu *et al.* (2010) and Luu-The *et al.* (2009). X, not detected; ✓, detected; ND, not done; and NR, not reported.

e.g., alcohol dehydrogenase 1B, amine oxidase, and epoxide hydrolase 1, were not detected in epidermal-derived HaCaT cells, but this was expected because these are predominantly dermally located enzymes (Luu-The *et al.*, 2009). This example demonstrates that when different models are compared, epidermal models should be compared with the epidermis, fibroblast models should be compared with the dermis, and full-thickness models should be compared with each other and *ex vivo* full-thickness native human skin. However, there were some enzymes, e.g., AKR 1B and catechol *o*-methyltransferase (COMT), which were detected in HaCaT cells but not in whole skin. This finding is of interest because AKR 1B mRNA is absent, and COMT is only present at a low level in whole skin (Hu *et al.*, 2009; Luu-The *et al.*, 2009).

Unlike phase I activities, phase II activities were readily detected in NHEK, HaCaT, and NCTC cells using plate reader methods (Götz *et al.*, 2012a,b), which is in line with the fact that skin tends to have relatively higher phase II than phase I XME capacity (Hu *et al.*, 2010; Oesch *et al.*, 2007). Of the phase II enzyme activities measured in the Cosmetics Europe projects, glutathione S-transferase (GST) is considered one of the most important (along with N-acetyltransferase [NAT]) in the detoxification of contact allergens (Schnuch *et al.*, 2011). The abundance of GST pi and GST omega proteins in

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HaCaT cells was equivalent to that in whole native skin, and these cells were also shown to metabolize the broad-spectrum GST substrate, 1-chloro 2,4-dinitrobenzene (CDNB) at the same rate as NHEKs and NCTC cells. The highest protein abundance was the GST pi isoform, which is in accordance with others who found this to be the major form in human skin (Blacker *et al.*, 1991). In contrast to native human skin, GST alpha and mu proteins were not detected in HaCaT cells although these have been shown to be lacking in primary keratinocyte cultures by others (Blacker *et al.*, 1991). NAT10 protein was detected in HaCaT cells, but NAT1 protein was extremely difficult to identify considering the readily measurable activity of this phase II enzyme in HaCaT cells (Götz *et al.*, 2012b). The lack of detection of NAT1 was not due to the detection method *per se* because spiked samples with recombinant NAT1 showed an LOD of 3 pmol/mg cytosolic protein.

3D Culture Models

There are several human 3D reconstructed skin (RS) models available, including the EpiSkin, EpiDerm, and SkinEthic RHE models. EpiSkin is validated as a stand-alone test and EpiDerm as a part of a testing strategy for skin irritation (Macfarlane *et al.*, 2009). The EpiDerm skin model has been incorporated into the RSMN assay and has so far proved to be promising for evaluating genotoxicity of dermally applied chemicals (Aardema *et al.*, 2010; Hu *et al.*, 2009; Mun *et al.*, 2009). The SkinEthic RHE model has been endorsed by the ECVAM Scientific Advisory Committee for distinguishing between corrosive and noncorrosive chemicals (de Brugerolle de Fraissinette *et al.*, 1999).

In the Cosmetics Europe project, proteins of the CYP 1–3 families were not detected in whole human skin, which reflects the low mRNA expression level of these CYPs reported by others (Luu-The *et al.*, 2009). Using recombinant CYPs to determine the detection sensitivity levels, the abundance of CYP1-3 proteins in human whole skin was estimated to be at levels at least 300-fold lower than those in liver. However, there were multiple other phase I XME proteins that were present in significant levels. These included alcohol dehydrogenases, aldehyde dehydrogenases, amine oxidases, and epoxide hydrolases (Table 2).

EpiDerm and EpiSkin models have been shown to exhibit a metabolic capability similar to native human skin, at least with respect to the mRNA expression of these enzymes (Hu *et al.*, 2010; Luu-The *et al.*, 2009). At the protein level, there were also some XMEs that were present in whole skin and in the three RS models tested (EpiDerm, EpiSkin, and SkinEthic RHE), for example, COX-2, catalase, alcohol dehydrogenase 3, and aldehyde dehydrogenases 2. Some phase I enzymes were absent in all the RS models, probably due to them being strictly epidermal models and the XMEs being predominantly dermally located, e.g., alcohol dehydrogenase 1B, epoxide hydrolase 1, and amine oxidase (Luu-The *et al.*, 2009). The knowledge that some XMEs are not detected in 3D RS models will help to interpret data from assays with compounds that require metabolic activation or detoxification via these pathways. It should also be kept in mind that whole skin consists of a dermis containing fibroblasts and an epidermis containing keratinocytes, which will interact with each other and therefore affect the level of enzymes in both cell types. This has been demonstrated with respect to CYP26B1 involved in *all-trans*-retinoic acid metabolism, which was absent in the epidermal model, EpiSkin, but present in its full-thickness skin model version (Luu-The *et al.*, 2009). The influence of the presence of fibroblasts on the XME expression in keratinocytes and the origin of the cells (male/female, anatomical site, etc.) makes a comparison with whole skin and between models difficult. For example, a few phase I enzymes located in the epidermis in whole skin were lacking in RS models, e.g., alcohol dehydrogenase 4 and aldehyde dehydrogenase 1L1, but this may be due to the absence of fibroblasts and the release of factors, which may be required for the expression of these XMEs in keratinocytes. Notably, the EpiSkin model expressed aldehyde dehydrogenase 3A2 protein, whereas the EpiDerm and SkinEthic RHE models did not. The reason for this difference between two epidermal models is not known although it is of interest that the EpiSkin is derived from female breast skin, whereas the EpiDerm and SkinEthic RHE models are derived from male foreskin. By contrast, there were some phase I enzyme proteins that were only expressed in RS models and not in whole skin. These included AKR 1B, which was detected in all RS models, and aldehyde dehydrogenase 7A1, which was detected in EpiDerm and RHE models but not in EpiSkin models. The mRNA expression of aldehyde dehydrogenase 7A1 in EpiDerm models is reported to be present at similar levels to that in adult human skin. The relatively high expression of aldehyde dehydrogenase 7A1 mRNA in human skin reported by Hu *et al.* (2010) would suggest that this protein should have been detected in whole skin. The reason for this discrepancy is unknown although there was a difference in skin sample site between the two studies (buttock and breast).

Of the phase II enzymes investigated, GST proteins were the most abundantly detected ones in both whole skin and EpiDerm models. Moreover, GST Pi was also identified as the most abundant isoform, which is known to be the most highly expressed GST in skin (Luu-The *et al.*, 2009) and is expressed at the mRNA level in the EpiDerm model used in our studies (Hu *et al.*, 2010). In addition to the GST protein being detected, it was also shown to be functional in the whole skin and RS models tested in the Cosmetics Europe metabolism project. The rate of metabolism of CDNB was appreciable in whole skin (~100 nmol/min/mg cytosolic protein). Moreover, CDNB metabolism was threefold higher in EpiDerm cytosol than that in whole-skin cytosol, which could be attributed to the regulating effect of both fibroblasts and keratinocytes on their gene expression.

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One of the most surprising findings of the Cosmetics Europe metabolism project was the very low abundance of NAT proteins in whole skin and RS models although their functional activities were clearly measureable by us and by others (Nohynek *et al.*, 2005). In fact, only one NAT isoform protein was detected, namely NAT10, and this was only in the EpiSkin model. The NAT1 protein was not detected in whole skin or any of the RS models. However, the mRNA expression of this phase II enzyme is also reported to be low in comparison with GST expression level in native human skin, EpiDerm, and EpiSkin models (Hu *et al.*, 2010; Luu-The *et al.*, 2009). Possible explanations for this discrepancy could be that (1) NAT1 is translated at a very low level, resulting in a protein abundance below the LOD of the proteomic technique, (2) the turnover of mRNA is rapid, or (3) this enzyme could be highly efficient in its catalytic activity such that the amount of NAT protein is relatively low compared with other enzymes, e.g., GSTs.

Sulfotransferase 1B1 (SULT1B1) mRNA is highly expressed in human skin and is predominantly located in the epidermis (Luu-The *et al.*, 2009). This enzyme sulfonates 3β-hydroxysteroids and cholesterol and is suggested to play a role in maintenance of the epithelial barrier (due to its association with cholesterol sulfate) and the granular-stratum corneal junction (Falany *et al.*, 2006; Higashi *et al.*, 2004). SULT1B1 protein was detected in all three RS models but not in whole skin. Hu *et al.* (2010) and Luu-The *et al.* (2009) both reported the overexpression of SULT1B1 mRNA in EpiDerm and EpiSkin models compared with whole skin, respectively, which may explain why this protein was only measurable in RS models and at a level below the LOD of the proteomic analysis in native skin.

Another phase II enzyme that was detected in RS models but not in whole skin was COMT. As for SULT1B1 mRNA, this is more highly expressed in EpiDerm and EpiSkin models than in whole skin (Hu *et al.*, 2010; Luu-The *et al.*, 2009), which may also explain the difference in the ability to detect the protein. UDP-glucuronosyltransferase (UGT) proteins were not detected in whole skin or any of the RS models that correlate with the low level of expression of these enzymes reported to be present in human skin (Hu *et al.*, 2010; Luu-The *et al.*, 2009). Although mRNA and protein expression levels were low, UGT activity measured using the broad-spectrum substrate, 4-methylumbelliferone, was similar in microsomes prepared from whole skin and the EpiDerm model (~1–1.8 nmol/min/mg protein), and both were similar to the activity measured in human liver microsomes (1–2.5 nmol/min/mg protein [characterization table at www.celsis.com]). Despite the high metabolism of 4-methylumbelliferone, care should be taken when considering the metabolism of potentially isoform-selective metabolism of chemicals, which (considering the low expression of some isoforms) could be much lower than that measured for the broad-spectrum substrate.

Application of Metabolic Data to Interpret the RSMN Assay

As mentioned previously, information on metabolic enzymes present in RS models can help to interpret the outcome of an *in vitro* assay. With this in mind, we investigated the XME profile of the EpiDerm model and whether it could change during the course of an endpoint assay. At this stage of the Cosmetics Europe metabolism project, there was a concomitantly running project investigating the genotoxicity of a number of metabolically activated chemicals using the RSMN assay (Aardema *et al.*, 2013); therefore, we measured a number of phase I and II enzyme activities using an EpiDerm model made of keratinocytes from the same donor and treated with either acetone or the progenotoxins, cyclophosphamide, and benzo[a]pyrene (B[a]P) (Götz *et al.*, 2012c). Cyclophosphamide is mainly metabolized by CYP2B6 although the EpiDerm model lacks this enzyme at the mRNA (Hu *et al.*, 2010), protein, and functional activity levels; this chemical was positive in the RSMN assay (Aardema *et al.*, 2010). Other CYPs such as CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP3A4, and CYP3A5 (Xie *et al.*, 2003) are reported to be involved in the bioactivation of cyclophosphamide in the liver. Thus, it is possible that CYP3A4/5 contributes to the bioactivation of this chemical in the RSMN assay because this CYP activity was detected at a low but measurable level in the EpiDerm model (Götz *et al.*, 2012c). Interestingly, the level of CYP3A4/5 in the EpiDerm model was not affected by cyclophosphamide, suggesting that this chemical did not induce its own metabolism via these CYPs. However, this theory was not investigated further by identifying and measuring the metabolites of cyclophosphamide and comparing the pattern obtained with that in the liver.

The outcome of the RSMN assay for B[a]P was more variable, such that one experiment resulted in a significant increase in micronuclei that was not reproduced in a second experiment (Aardema *et al.*, 2013). The bioactivation of B[a]P is mainly by CYP1B1, CYP1A1, and microsomal epoxide hydrolase (Hvastkovs *et al.*, 2007), and the ultimate genotoxin is detoxified by GSTs and UGTs (Mackenzie *et al.*, 1993; Upadhyaya *et al.*, 2010). Costa *et al.* (2010) showed that CYP1A1 mRNA is induced in human skin by B[a]P, and considering that modulation of CYPs alters the amount of DNA adducts formed (Wei *et al.*, 2009); it was important to demonstrate whether this effect was also observed in B[a]P-treated EpiDerm model and whether this could explain the variable genotoxic response. EROD activity was increased by B[a]P treatment by 10-fold above acetone treated models; however, CYP1A proteins were still not detected at the protein level in B[a]P-treated models. This could be expected considering that the levels in skin were estimated to be over 300-fold lower than those in the liver, such that an induction of 10-fold would not bring the abundance of these CYPs above the LOD for the proteomic method used in these studies. Moreover, even with the significant induction of CYPs, the level of bioactivation would remain lower than the detoxification pathways via GSTs and UGTs, such

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that detoxification would always be the predominant pathway at the concentrations used in this experiment. Although B[a]P significantly induced EROD activity, even at the lowest dose tested in the RSMN assay, at all three time points tested (up to 72 h), the opposing detoxification UGT and GST pathways were not similarly induced, possibly because, unlike CYP1, they were already at relatively high levels. These data suggest that the level of CYP1A and its induction are not the sole factors affecting the outcome of the genotoxicity of B[a]P in the EpiDerm model. This is supported by Kim *et al.* (1997) who demonstrated that peroxidases are involved in the bioactivation of B[a]P.

SUMMARY AND CONCLUSIONS

The aim of the projects described herein was to characterize the metabolic capacity of different 2D and 3D models in order to help interpret data generated from assays upon which they are based. The different models compared here are already used for predicting various endpoints, such as sensitivity and genotoxicity, and these have been used regardless of any knowledge of the metabolizing enzymes that they contain because they have been tested in validation studies for their suitability for a selected toxic endpoint and shown to be adequate for that purpose. Data generated by the Cosmetics Europe xenobiotic metabolism projects have provided important information on the metabolic profiles of native human skin and some of the *in vitro* models used by the cosmetics industry for various toxicity endpoints. Knowledge of a difference between an *in vitro* model and native human skin should not exclude the use of the *in vitro* model (especially if it predicts the endpoint) rather, the difference in the metabolism could be taken into account when interpreting the outcome or extrapolating the findings to native human skin. For example, measurement of phase I and II activities under the conditions of the RSMN assay suggests that levels of metabolizing enzymes may change during the course of an assay and that a chemical may induce its own bioactivation. The induction effect of B[a]P demonstrated here in the EpiDerm model has also been shown to occur in *ex vivo* human skin (Costa *et al.*, 2010), supporting the use of the RS model in this kind of assay as an alternative to fresh human skin although more extensive comparisons will be needed to determine how comparable the induction capacities are.

The use of proteomics and functional assays allowed for an overall assessment of the types of XMEs present in these models, as well as more in-depth investigations of selected phase I and II activities. Figure 1 provides an overview of the potential routes of metabolism of xenobiotics in the skin and how these are comparable with the liver. XME activities are very low in skin compared with those in liver. Nevertheless, it is also pertinent to consider the comparatively larger size of the skin when considering the overall capacity for metabolism of dermally applied compounds as under some circumstances the low levels of XME may still contribute. This is especially relevant to compounds or products that are applied to the entire

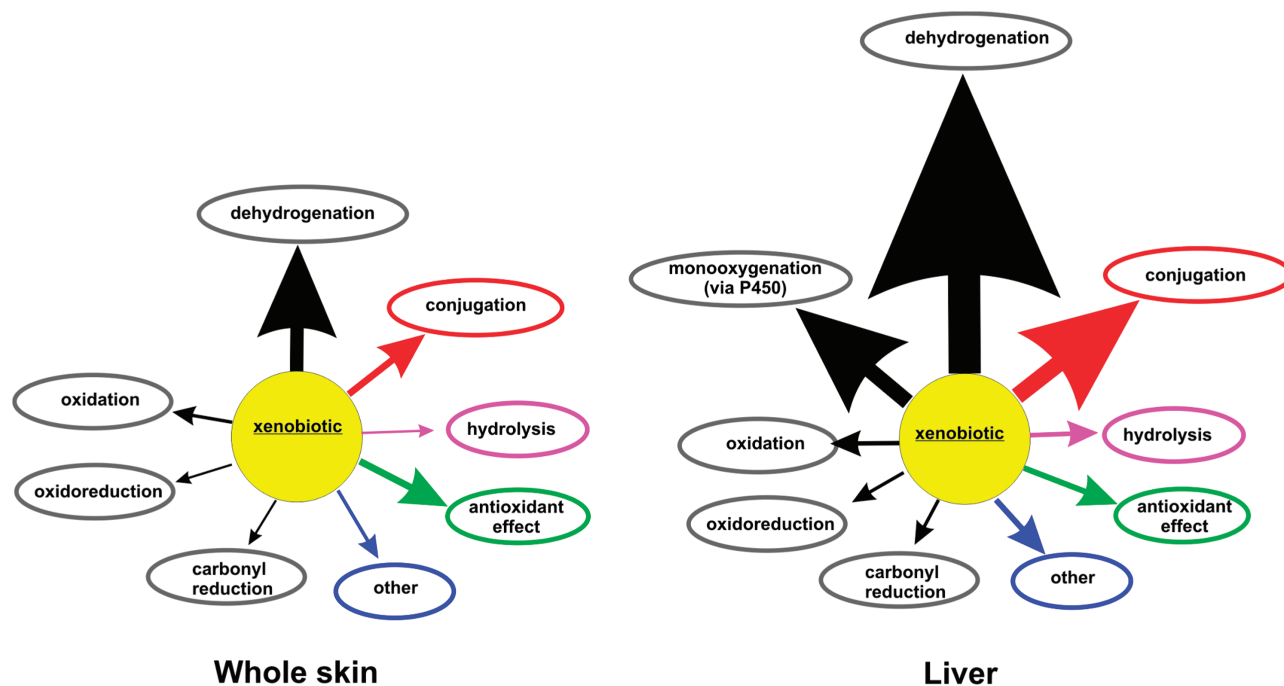

FIG. 1. Potential routes of xenobiotic metabolism in skin and liver. The size of each arrow is proportional to the number of XMEs detected that may catalyze each bioconversion indicated (van Eijl *et al.*, 2012).

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body, such as bath products and sun lotions, which penetrate the stratum corneum. However, in cases where the portion of the skin to which the compound or product is applied is smaller (e.g., face cream), the total metabolic capacity will be reduced accordingly because topically applied compounds can only be metabolized by the enzymes present in exposed skin.

Some XMEs in whole skin were not detected in *in vitro* models and *vice versa*, and some major hepatic XMEs such as CYPs were absent or measured only at very low levels in the skin. EROD activity, attributed to CYP1A, seemed to be inducible by classic polycyclic aromatic hydrocarbon inducers (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 3-methylcholanthrene, B[a]P). Their level of activity reached after induction was still very low compared with that observed in the liver but could still be sufficient for bioactivation of progenotoxins and sensitizers although this has to be investigated further. The contribution of other phase I enzymes, such as peroxidases, may also be considered as potential alternative pathways of bioactivation. Clearly, predominantly dermal XMEs are not expected to be present in epidermal models but there were a number of enzymes that were not detected in RS models, which were present in whole skin. The known low expression and activity of phase I enzymes in native whole skin were also reflected in the *in vitro* models, and despite varying mRNA and protein levels of phase II enzymes, functional activities of GSTs, NAT1, and UGTs were all readily measurable in whole skin and 2D and 3D *in vitro* skin models at activity levels similar to those measured in the liver. Therefore, it should be kept in mind that the abundance of a protein does not necessarily reflect its activity.

In conclusion, the characterization of phase I and II enzymes in *in vitro* human models used as relevant alternatives to *in vivo* assays for sensitization and genotoxicity allows for a better interpretation of the data. Moreover, the comparison of enzymes in these models with native human skin suggests that they can be considered to possess similar properties of xenobiotic metabolism, which will help in extrapolating the outcomes of the assays to *in vivo*.

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