

Xenobiotic metabolism gene expression in the EpiDerm™ *in vitro* 3D human epidermis model compared to human skin

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ABSTRACT

There is an urgent need to validate *in vitro* human skin models for use in safety testing. An important component of validation is characterizing the metabolizing capacity of these models. We report comparison of the expression of 139 genes encoding xenobiotic metabolizing enzymes in the EpiDerm™ model and human skin. In microarray analysis, the expression of 87% of the genes was consistent between the EpiDerm™ model and human skin indicating the presence of similar metabolic pathways suggesting commonality in function. Analysis of EpiDerm™ models constructed from four donors showed highly comparable expression of xenobiotic metabolizing genes demonstrating reproducibility of the model. Overall, the expression of Phase II enzymes appeared to be more pronounced in human skin and the EpiDerm™ model than that of Phase I enzymes, consistent with the role of skin in detoxification of xenobiotics. Though the basal expression of CYPs in particular was low in EpiDerm™, significant induction of CYP1A1/1B1 activity was observed following treatment with 3-methylcholanthrene. These results indicate that the xenobiotic metabolizing capacity of the EpiDerm™ model appears to be representative of human skin. Models such as EpiDerm™ provide a valuable *in vitro* approach for evaluation of metabolism and toxicity of cutaneous exposures to xenobiotics.

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1. Introduction

Human skin provides an important protective barrier against potentially harmful physical and chemical exposures via its outermost layer, the non-viable and hydrophobic stratum corneum. Skin is also a metabolically competent organ involved in the activation and detoxification of chemicals that have passed through the stratum corneum. Skin is therefore critical with respect to both local and systemic toxicity from dermal exposures, a major route of exposure for many products including cosmetics, chemicals, and drugs. Indeed, the skin plays a key role in controlling the penetra-

Abbreviations: 3D, three-dimensional; 3-MC, 3-methylcholanthrene; AKR, 3 α -hydroxysteroid dehydrogenase; ALDH, aldehyde dehydrogenase; CES, carboxylesterase; EROD, ethoxyresorufin O-deethylase; FTHBS, full thickness human buttock skin; GPX, glutathione peroxidase; GST, glutathione S-transferase; PAPSS, 3'-phosphoadenosine 5'-phosphosulfate synthase; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcription PCR; RSMN assay, Reconstructed Skin MicroNucleus assay; UGT, UDP-glucuronosyltransferase; XB, xenobiotic.

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tion and metabolism of topically applied chemicals. However, despite its importance, the role of cutaneous metabolism in toxicity and the current extent of characterization of xenobiotic (XB) metabolism in normal human skin is limited (Gibbs et al., 2007; Oesch et al., 2007). As recently reviewed by Gibbs et al. (2007) and Oesch et al. (2007), most drug metabolizing enzymes including Phase I (e.g. cytochrome P450s, esterases) and Phase II (e.g. glutathione S-transferase, UDP glucuronidation) pathways are present in skin, more specifically in the epidermis, with higher amounts occurring in the keratinocytes compared to other cell types. Despite the fact that most metabolizing enzymes are present in skin, the activity is typically lower than liver though the metabolic activity of skin is still considered relevant from a toxicological standpoint. The reader is referred to the above extensive reviews for more discussion on human skin metabolism.

Human skin explants are conventionally used to study processes including XB metabolism in human skin and skin absorption. However, the use of human tissue has a number of disadvantages, including difficulty in obtaining fresh human skin for immediate experimental use and source-dependent variability in tissue handling and tissue quality that can affect the level of XB metabolizing enzymes. The variability and limited availability of

human skin samples, along with the fact that most metabolic capacity is present in epidermal keratinocytes, have led various investigators to use *in vitro* keratinocyte cultures and *in vitro* 3D skin models to study the metabolic behavior of human skin, as well as efficacy and safety of chemical and product exposure. For example, three-dimensional skin models have recently been used for the *in vitro* evaluation of topically applied chemicals with respect to various characteristics (Roguet, 2004), including cutaneous irritancy (Welss et al., 2004), percutaneous absorption (Schafer-Korting et al., 2006), and cutaneous XB metabolism of specific chemicals (Nohynek et al., 2005; Hu et al., 2009a). Overall, studies to date are still relatively limited, but indicate that major metabolic pathways are present in these *in vitro* systems. It is recognized that there may be some inherent differences between these *in vitro* systems and skin because of various technical factors (e.g. influence of cell culture conditions) and it is not expected that they will fully reflect the *in vivo* situation (see review of Gibbs et al., 2007). More work is clearly needed to define the similarities and differences between human skin and *in vitro* systems to better determine their limitations.

The effort to further develop and validate *in vitro* human skin models has become urgent following the enactment of the Registration, Evaluation, Authorization and restriction of CHemicals (REACH) legislation in June 2007 and the 7th Amendment to the EU Cosmetics Directive in March 2009. In particular, the 7th Amendment prohibits animal testing of cosmetic ingredients, including the use of animals for skin irritation, corrosion, and genotoxicity testing, as of March 2009. Furthermore, the use of animals for ADME testing of cosmetic ingredients will be banned from March 2013. For genotoxicity testing, conventional *in vitro* tests alone do not provide an appropriate assessment of hazard since it has been demonstrated that the standard initial battery of *in vitro* assays have a high level (75–95%) of false positive results (Kirkland et al., 2005). Since positive results *in vitro* are typically followed up by *in vivo* genotoxicity assays and these are no longer possible for cosmetic ingredients, there has been a focused effort to develop improved *in vitro* methods especially for evaluation of cosmetic ingredients.

To address the need for more predictive *in vitro* genotoxicity assays, we have developed (Curren et al., 2004, 2006) and characterized (Hu et al., 2009b; Mun et al., 2009) a novel micronucleus assay, the Reconstructed Skin MicroNucleus (RSMN) assay, using three-dimensional (3D) human skin EpiDerm™ constructs. The EpiDerm™ model is a three-dimensional multilayered skin culture derived from human infant foreskin keratinocytes that closely resembles human epidermis (Cannon et al., 1994). The RSMN assay in EpiDerm™ tissues has been shown to discriminate correctly between direct-acting genotoxic skin carcinogens and non-skin carcinogens (Hu et al., 2009b; Mun et al., 2009). An international collaborative effort, supported by the European Cosmetics Industry Association (COLIPA), to further evaluate and validate the 3D RSMN assay in EpiDerm™ is now under way. Other efforts to develop genotoxicity assays in 3D skin models have been reported, for instance Flamand et al. (2006) describe the development of a Comet assay in the EpiSkin® model.

If *in vitro* reconstructed epidermis models are to be used to predict responses to cutaneous XB exposures they must express the multitude of XB metabolizing pathways present in human skin and for genotoxicity assays, they must appropriately detect genotoxins that require metabolism. Initial studies from our laboratories indicate that EpiDerm™ cultures metabolize XBs, as indicated by positive results for chemicals that require metabolic activation in the RSMN assay (Kaluzhny et al., 2008; Aardema et al., in preparation). Hu et al. (2009a) demonstrated N-acetylation of the hair dye constituents *p*-aminophenol and *p*-phenylenediamine by EpiDerm™ constructs, in agreement with studies using

EpiSkin® (Nohynek et al., 2005) and normal human scalp (Nohynek et al., 2004).

Further work is urgently needed to demonstrate that these important 3D skin models have XB metabolism pathways representative of those in human skin. In this paper, we describe the analysis of gene expression in 3D EpiDerm™ *in vitro* models compared to normal full thickness human buttock skin (FTHBS). Importantly, we analyzed EpiDerm™ models prepared from a variety of donors since the donor used in the construction of the model changes over time. While protein expression levels and functional assays are preferable for the assessment of gene activity, these are technically challenging in skin requiring sensitive analytical techniques and are not amenable to studying large numbers of gene products and samples. Our studies therefore focused on genome-wide microarray analysis since it provides relative quantification of the entire mRNA population and therefore offers an overview in this case, of the expression of the multitude of genes involved in metabolic pathways in skin. This is a first, and essential step in characterizing the metabolic capacity in human skin and the EpiDerm™ model. Since expression of mRNA may not be proportional to functional activity, further detailed studies at the protein and functional level are needed to confirm the metabolic activity of human skin and skin models.

We identified 139 major genes involved in Phase I and Phase II reactions for the biotransformation of XBs according to the functional classification of Cassarett and Doull (Klaassen, 2008). We evaluated expression of these genes and verified the results for key genes by quantitative reverse transcription polymerase chain reaction (PCR). Induction of various cytochrome P450s (CYPs) was measured at the mRNA level and functional assays for CYP1A1 (ethoxyresorufin O-deethylase; EROD), UDP-glucuronosyltransferase (UGT; 4-methylumbelliferone metabolism) and glutathione S-transferase (GST; 1-chloro-2,4-dinitrobenzene conjugation) were conducted. These results provide an overview of the metabolic potential of the EpiDerm™ model compared to human skin and its consequent applicability as a non-animal method for determining the efficacy, metabolism, and toxicity of cutaneous exposures.

2. Materials and methods

2.1. Reconstructed EpiDerm™ human epidermis model

Reconstructed human EpiDerm™ EPI-200 tissues prepared from four different neonatal foreskin donors (254, 1188, 219 and 926) were acquired from MatTek Corporation (Ashland, MA). For Affymetrix analyses, five tissues from each donor were placed into 6-well plates with 1 ml/tissue/well of pre-warmed (37 °C) NMM media (MatTek Corporation) and incubated overnight at 37 °C. The following day, individual epidermal tissues were peeled from the tissue insert and placed into 2 ml of RNAlater® (Ambion/ABI, Foster City, CA) pre-loaded into a 24-well plate. After 24 h incubation at 4 °C, the RNAlater® submersed tissues were transferred into 2 ml Seal-Rite® microcentrifuge tubes (USA Scientific Inc., Ocala, FL) and all excess RNAlater® was removed from the tissue by gentle pipetting. The tissues were stored at –80 °C until RNA was isolated.

For the studies performed at MatTek Corporation, all EpiDerm™ tissues were packaged and stored at 4 °C for 24 h to simulate shipping. Tissues were then placed into 6-well plates with 1 ml/tissue/well of NMM media and incubated at 37 °C for 24 h before any experiments were started. For reverse transcription PCR experiments and GST activity assay, three EpiDerm™ Donor 254 cultures were treated with 3-methylcholanthrene (3-MC; 10 µM) for 24 h, three cultures were treated with 0.1% (v/v) acetone, and three were untreated. For the UGT activity assay and EROD activity assay, three EpiDerm™ cultures from each of the four donors for each

condition (3-MC (10 μ M) for 48 h; 0.1% (v/v) acetone, and untreated) were used. For qRT-PCR studies, one culture per donor of the RNA isolated for the Affymetrix microarrays was used.

2.2. Human skin sample preparations

All human skin samples were obtained with Institutional Review Board approval and with written informed consent in accordance with the Declaration of Helsinki principles. One full thickness biopsy (4 mm) was collected from buttocks of each of 10 female subjects (18–20 years of age). Each biopsy was placed directly into RNAlater[®] and stored overnight at 4 °C, removed from RNAlater[®], then stored at –80 °C until RNA isolation. For qRT-PCR arrays performed at MatTek, full thickness 5 cm \times 5 cm samples from breast skin were obtained from two donors, one from a 26 year old and one from a 64 year old female undergoing elective cosmetic surgery. Subcutaneous fat was removed and total RNA was isolated from the full thickness breast skin using the RNeasy[®] isolation kit (Ambion/ABI, Foster City, CA).

2.3. Total RNA for Affymetrix expression profiling

Total RNA was extracted and purified following the procedure described by Naciff et al. (2009). In brief, total RNA was isolated and purified from each sample using Trizol (Invitrogen, Carlsbad, CA) and RNeasy kits (Qiagen, Valencia, CA). Total RNA from full thickness human buttock skin (FTHBS) (5 μ g per sample) was converted into double-stranded cDNA using SuperScript Choice system (Invitrogen Corporation/GIBCO BRL, Rockville, MD) with an oligo-dT primer containing a T7 RNA polymerase promoter and further purified by phenol/chloroform extraction followed by *in vitro* transcription using the ENZO BioArray RNA transcript labeling kit (Enzo Life Sciences, Inc., Farmingdale, NY). Total EpiDerm[™] tissue RNA (1 μ g per sample) from four different donors was converted to biotin-labeled double-stranded cDNA using Ambion Message Amp II[–] Biotin Enhanced Kit according to manufactures recommendations. Biotin-labeled cRNA was purified by the RNeasy kit (Qiagen), and a total of 20 μ g of cRNA were fragmented randomly to ~200 bp at 94 °C for 35 min (200 mM Tris–acetate, pH 8.2, 500 mM KOAc, 150 mM MgOAc). An Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA) was used to determine the target cRNA quality and samples from five individual sample replicates, from each treatment group with high quality cRNA, were selected and hybridized to Affymetrix Human Genome U133 Plus 2.0 high-density oligonucleotide microarrays (Affymetrix Inc., Santa Clara, CA) for 16 h. Samples from EpiDerm[™] were all hybridized the same day using the same lot of chips. The microarrays were washed and stained by streptavidin–phycoerythrin to detect bound cRNA. The signal intensity was amplified by second staining with biotin-labeled anti-streptavidin antibody followed by streptavidin–phycoerythrin staining. Fluorescent images were read using the Affymetrix GeneChip Scanner 3000 with Autoloader (Affymetrix Inc., Santa Clara, CA).

2.4. Reverse transcription PCR analysis

Total RNA was isolated from three EpiDerm[™] 254 cultures per treatment using RNeasy[®] and DNase^d with DNA-free (ABI/Ambion). Purified total RNA was quantified by UV spectroscopy and its quality analyzed by agarose gel electrophoresis. Using the ReactionReady[™] First Strand Kit (SA Biosciences/Qiagen, Frederick, MD), cDNA was synthesized from 1 μ g total RNA. Reverse transcription PCR of CYP mRNAs was performed using a commercially available kit containing optimized primers for 11 human CYP isozymes and a housekeeping gene (Human P450 Gene Family I MultiGene-12[™] reverse transcription PCR Profiling Kit: PH-027B,

and ReactionReady[™] HotStart “Sweet” PCR master mix, SA Biosciences/Qiagen). The reactions were run on a Eppendorf (Westbury, NY) Mastercycler[®] Gradient thermocycler with a 10 min hot start at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. The PCR products were run on a 2% TBE–agarose gel and visualized with ethidium bromide. The reverse transcription PCR experiment was repeated three times, and representative gel images were selected.

2.5. Quantitative reverse transcription PCR analysis

qRT-PCR arrays containing optimized primers for Phase I or Phase II genes, five reference genes, and seven internal controls were used (Human Drug Metabolism: Phase I Enzymes RT² Profiler[™] PCR Array, Human Drug Metabolism: Phase II Enzymes RT² Profiler[™] PCR Array and RT² SYBR[®] Green qPCR Master Mix, SA Biosciences/Qiagen). For each of the four EpiDerm[™] donors, 1 μ g total RNA from one culture of the same RNA previously isolated for the Affymetrix microarrays was used; 1 μ g RNA from each of the two MatTek human full thickness breast skin samples was used. Each RNA sample was treated with DNase using a DNA-free kit (Ambion/ABI, Foster City, CA), quantitated by UV spectrophotometry, and analyzed by agarose gel electrophoresis to verify RNA quality. The RT² First Strand Kit (SA Biosciences/Qiagen, Frederick, MD) was used for cDNA synthesis.

Each array was run on a CFX96[™] real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) with a 10 min hot start at 95 °C, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. A dissociation curve was run after 40 cycles to verify single product formation. The arrays were run in triplicate, and analyzed according to the Delta Ct method with the accompanying RT² Profiler[™] PCR Array Data Analysis software (SA Biosciences/Qiagen). Results from the triplicate arrays for each EpiDerm[™] sample were averaged. Results for the two human tissue samples were averaged. Any gene with a Ct value of 35 or more was considered absent. Fold changes were calculated by the RT² Profiler[™] PCR Array Data Analysis Software.

2.6. Functional classification of Phase I and Phase II enzymes involved in the biotransformation of XBs

Genes involved in Phase I and Phase II XB biotransformation reactions were identified according to the functional classification of Cassarett and Doull (Klaassen, 2008), followed by specific text queries from updated public gene annotation sources, such as: the Gene Ontology Consortium, (<http://www.geneontology.org>), Swiss-Prot (<http://www.ebi.ac.uk/swissprot/>), Affymetrix (www.affymetrix.com), Entrez (<http://www.ncbi.nlm.nih.gov/sites/gquery>), and HUGO (<http://www.genenames.org/>). The resulting gene names were sorted into categories, as shown in Tables 1 and 3, from enzymes that participate in Phase I and Phase II reactions involved in the biotransformation of XBs.

2.7. Selected XB genes: microarray data analysis

GeneChip Analysis was performed using the Affymetrix MAS5.0 algorithm (www.affymetrix.com). This algorithm is used to generate signal values and “Present” (P) or “Absent” (A) calls (“Marginal” calls were classified as P in our analyses). For genes with redundant probe sets, the Affymetrix probe set with the highest average signal intensity level in FTHBS was selected to represent the gene. Selected probe sets with Affymetrix “P” calls between 30% and 40% among the replicates (2/5 for each replicate obtained from each of the four EpiDerm[™] donors and 3/10 for human buttocks) were considered as present. The FTHBS dataset served as the basis for the calculation of Affymetrix signal intensity ratio. Negative signs

Table 1

Classification of XB metabolizing genes as “present” or “absent” in full thickness human buttock skin and EpiDerm™ tissues.

Gene name	Affymetrix ID	Affymetrix present and absent call				
		FTHBS	254	1188	219	926
(a) Phase I cytochrome P450-dependent oxidation enzymes						
Cytochrome P450-dependent monooxygenases						
CYP1A1	205749_at	P	A	A	A	A
CYP1A2	207608_x_at	P	P	P	P	P
CYP1B1	202436_s_at	P	P	P	P	P
CYP2A6	1494_f_at	P	A	A	A	A
CYP2A7	207718_x_at	A	A	A	A	A
CYP2A13	208327_at	A	A	A	A	A
CYP2B6	206754_s_at	A	A	A	A	A
CYP2C8	208147_s_at	A	A	A	A	A
CYP2C9	216025_x_at	P	P	P	P	P
CYP2C18	208126_s_at	P	P	P	P	P
CYP2C19	216058_s_at	A	A	A	A	A
CYP2D6	207498_s_at	P	A	P	P	P
CYP2E1	209975_at	P	A	A	A	A
CYP2F1	207913_at	A	A	A	A	A
CYP2J2	205073_at	P	P	P	P	P
CYP2R1	227109_at	P	P	P	P	P
CYP2S1	223385_at	P	P	P	P	P
CYP2U1	226393_at	P	P	A	P	P
CYP2W1	220562_at	P	A	P	A	P
CYP3A4	205998_x_at	A	A	A	A	A
CYP3A5	214235_at	P	P	P	P	P
CYP3A7	211843_x_at	A	A	A	A	A
CYP3A43	211440_x_at	P	A	P	A	A
CYP4A11	1554837_a_at	P	A	A	A	P
CYP4A22	217319_x_at	A	A	A	A	A
CYP4B1	1555497_a_at	P	P	P	P	P
CYP4F2	210452_x_at	A	A	A	A	A
CYP4F3	206515_at	P	P	P	P	P
CYP4F8	210576_at	P	P	P	P	A
CYP4F11	206153_at	A	A	A	A	A
CYP4F12	206539_s_at	P	P	P	P	P
CYP4F22	244692_at	P	P	P	P	P
CYP4V2	226745_at	P	P	P	P	P
CYP4X1	227702_at	P	A	A	A	A
CYP4Z1	237395_at	A	A	A	A	A
CYP4Z2P	1553434_at	A	A	A	A	A
CYP7A1	207406_at	A	A	A	A	A
CYP7B1	207386_at	P	P	P	P	P
CYP8B1	232494_at	P	A	A	A	A
CYP11A1	204309_at	A	A	A	A	A
CYP11B1	214610_at	A	A	A	A	A
CYP11B2	214630_at	A	A	A	A	A
CYP17A1	205502_at	A	A	A	A	A
CYP19A1	240705_at	A	A	A	A	A
CYP20A1	219565_at	P	P	P	P	P
CYP21A2	214622_at	A	A	A	A	A
CYP24A1	206504_at	A	P	P	A	P
CYP26A1	206424_at	A	A	A	A	A
CYP26B1	219825_at	P	P	P	P	P
CYP27A1	203979_at	A	A	A	A	A
CYP27B1	205676_at	P	P	P	P	P
CYP27C1	1568868_at	P	P	P	P	P
CYP39A1	1553977_a_at	P	P	P	A	A
CYP46A1	220331_at	A	A	A	A	A
CYP51A1	216607_s_at	P	P	P	P	P
Prostaglandin I2 (prostacyclin) synthase						
PTGIS	208131_s_at	P	A	A	A	A
Thromboxane A synthase						
TBXAS1	208130_s_at	A	A	A	A	A
(b) Other Phase I enzymes						
Oxidases						
Aldehyde dehydrogenase						
AKR1A1	201900_s_at	P	P	P	P	P
AKR1C1	204151_x_at	P	P	P	P	P
AKR1C3	209160_at	P	P	P	P	P
AKR1C4	210558_at	A	A	A	P	A
ALDH1A3	203180_at	P	P	P	P	P
ALDH1B1	209645_s_at	P	A	A	A	A
ALDH2	201425_at	P	P	P	P	P

Table 1 (continued)

Gene name	Affymetrix ID	Affymetrix present and absent call				
		FTHBS	254	1188	219	926
ALDH3A1	205623_at	P	P	P	P	P
ALDH3A2	202053_s_at	P	P	P	P	P
ALDH3B1	211004_s_at	P	A	A	A	A
ALDH3B2	204942_s_at	P	P	P	P	P
ALDH7A1	208950_s_at	P	P	P	P	P
Dihydrodiol dehydrogenase (dimeric)						
DHHDH	231416_at	A	A	A	A	A
Ferredoxin						
FDX1	203647_s_at	P	P	P	P	P
Glucose-6-phosphate dehydrogenase						
G6PD	202275_at	P	P	P	P	P
Nicotinamide nucleotide transhydrogenase						
NNT	202783_at	P	P	P	P	P
NAD(P)H dehydrogenase, quinone						
NQO1	210519_s_at	P	P	P	P	P
NQO2	203814_s_at	P	P	P	P	P
Sorbitol dehydrogenase						
SORD	201563_at	P	P	P	P	P
Flavin-containing monooxygenase						
FMO1	205666_at	P	A	P	A	A
FMO2	228268_at	P	P	P	P	P
FMO3	40665_at	P	A	A	A	A
FMO4	206263_at	P	P	P	P	P
FMO5	205776_at	P	P	P	P	P
Glutathione peroxidase						
GPX1	200736_s_at	P	P	P	P	P
GPX2	202831_at	P	P	P	P	P
GPX3	201348_at	P	P	P	P	P
Aldehyde oxidase						
AOX1	205083_at	P	A	P	P	A
Reductases						
Aldo-keto reductase						
AKR7A2	214259_s_at	P	P	P	P	P
AKR7A3	206469_x_at	P	P	P	P	P
Cytochrome b5 reductase						
CYB5R1	202263_at	P	P	P	P	P
CYB5R2	220230_s_at	P	P	P	P	P
CYB5R3	201885_s_at	P	P	P	P	P
Superoxide dismutase						
SOD2	221477_s_at	P	P	P	P	P
Hydrolytic enzymes						
Carboxylesterase						
CES1	209616_s_at	P	P	P	P	P
CES2	213509_x_at	P	P	P	P	P
CES3	220335_x_at	A	A	P	P	A
CES7	1553465_a_at	A	A	A	A	A
Epoxide hydrolase						
EPHX1	202017_at	P	P	P	P	P
EPHX2	209368_at	P	P	P	P	P
Glucosidase, beta, acid						
GBA3	219954_s_at	P	P	P	P	A
Kynureninase						
KYNU	204385_at	P	P	P	P	P
<i>(c) Phase II enzymes</i>						
Transferases						
Glutathione S-transferase						
GSTA1	215766_at	P	P	P	P	P
GSTA4	202967_at	P	P	P	P	P
GSTM1	215333_x_at	P	P	P	P	P
GSTM2	204418_x_at	P	P	P	P	P
GSTM3	202554_s_at	P	P	P	P	P
GSTM4	210912_x_at	P	P	P	P	P
GSTM5	205752_s_at	P	A	P	A	P
GSTP1	200824_at	P	P	P	P	P
GSTZ1	209531_at	P	P	P	P	P
UDP-glucuronosyltransferase						
UGT1A6	232654_s_at	P	P	P	P	P
UGT1A8	221305_s_at	A	P	P	P	P
UGT1A10	204532_x_at	P	P	P	P	P
UGT2A1	207958_at	P	A	A	A	A
UGT2A3	219948_x_at	A	A	A	P	A
UGT2B4	206505_at	A	A	A	A	A
UGT2B15	207392_x_at	A	A	A	A	A

(continued on next page)

Table 1 (continued)

Gene name	Affymetrix ID	Affymetrix present and absent call				
		FTHBS	254	1188	219	926
UGT2B17	207245_at	A	A	A	A	A
UGT2B28	211682_x_at	A	A	A	A	A
UGT3A1	236597_at	A	A	A	A	A
Galactose-3-O-sulfotransferase						
GAL3ST1	205670_at	A	A	A	A	A
Sulfotransferase						
SULT1A1	203615_x_at	P	P	P	P	P
SULT1A2	207122_x_at	P	A	A	A	A
SULT1A4	210580_x_at	P	P	P	P	P
SULT1B1	207601_at	A	A	A	A	A
SULT1C2	205343_at	A	A	A	A	A
SULT1C4	1553321_a_at	A	A	A	A	A
SULT1E1	219934_s_at	P	P	P	P	P
SULT2A1	206292_s_at	A	A	A	A	A
SULT2B1	205759_s_at	P	P	P	P	P
SULT4A1	219425_at	A	A	A	A	A
N-acetyltransferase						
NAT1	214440_at	P	P	P	P	P
NAT2	206797_at	A	A	A	A	A
Cysteine conjugate β -lyase						
CCBL1	206037_at	P	A	A	A	A
γ -Glutamyl transferase						
GGT7	226470_at	P	P	A	A	A
3'-Phosphoadenosine 5'-phosphosulfate synthase						
PAPSS1	209043_at	P	P	P	P	P
PAPSS2	203060_s_at	P	A	A	A	A
Catechol-O-methyltransferase						
COMT	208818_s_at	P	P	P	P	P
Thiopurine S-methyltransferase						
TPMT	203672_x_at	P	P	P	P	P
Ligases						
Glutamate-cysteine ligase, catalytic subunit						
GCLC	202923_s_at	P	P	P	P	P
Glutathione synthetase						
GSS	211630_s_at	P	P	P	P	P

(–) were employed to represent the ratio when the signal intensity level is less than that of human FTHBS.

2.8. Ethoxyresorufin O-deethylase activity assay

In order to determine whether functional CYP1A1 and/or CYP1B1 isoforms were expressed in EpiDerm™ tissues following induction with 3-MC, EROD activity was determined. The method used was that described by Harris et al. (2002b), with the following modifications: after 48 h induction with 3-MC, cultures were transferred to fresh medium (phenol-red free) containing 5 μ M ethoxyresorufin and 33 μ M dicumarol (to inhibit further metabolism of resorufin by NQO1, NADPH: quinone oxidoreductase) and incubated for 120 min at 37 °C. Samples (200 μ l) of the culture medium were taken, and resorufin fluorescence was measured at 530 nm excitation and 590 nm emission on a Biotek Synergy HT microplate reader.

2.9. UDP-glucuronosyltransferase activity assay

After 48 h induction with 3-MC (10 μ M), as described above, UGT activity was determined as described by Gomez-Lechon et al. (2001). Briefly, three EpiDerm™ cultures from each donor were incubated with 100 μ M 4-methylumbelliferone in the culture medium for 1 h at 37 °C. Samples of culture medium were diluted 1:20 in 10 mM NaOH and the remaining 4-methylumbelliferone was quantified fluorometrically at 376 nm excitation and 460 nm emission. The percentage of conjugated 4-methylumbelliferone was calculated from the percentage of the remaining unconjugated substrate.

2.10. Glutathione S-transferase activity assay

After 24 h, three EpiDerm™ Donor 254 cultures from each condition (3-MC treated, plus untreated and vehicle controls) were lysed in 5 ml of ice-cold 100 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA. Total GST activity in EpiDerm™ culture lysates was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione using a GST assay kit (Cayman Chemical, Ann Arbor, MI). This GST-mediated conjugation reaction results in an increase in absorbance at 340 nm. GST activity was expressed as nanomoles per minute per ml of solution.

3. Results

3.1. Affymetrix analysis of presence and absence of genes

The expression of 139 genes encoding Phase I and Phase II drug metabolizing enzymes was evaluated by Affymetrix microarray analysis in samples from FTHBS and in EpiDerm™ tissue from four different donors (designated 254, 1188, 219 and 926). Table 1 summarizes the presence or absence of expression of the 139 genes analyzed. Overall, excellent agreement was observed, 121 genes (87%) being consistent between EpiDerm™ and FTHBS; 83 genes were present in both EpiDerm™ and FTHBS, and 38 genes were present in neither tissue type (Fig. 1). Of 139 genes analyzed, only five (~3.5%) genes were unique to EpiDerm™ tissue (i.e. expressed in EpiDerm™ tissue and not in FTHBS) and only 13 genes were expressed in FTHBS but not in EpiDerm™ tissue (Fig. 1 and Table 1). The concordance between EpiDerm™ tissues from each of the four different donors and FTHBS was also analyzed and was high with 83–86% agreement (Table 2).

The five genes detected in EpiDerm™ tissues but not in FTHBS were CYP24A1, UGT1A8, UGT2A3, CES3 (carboxylesterase Type 3) and AKR1C4 (3 α -hydroxysteroid dehydrogenase, Type I). Only UGT1A8 was expressed in tissue from all four donors, CYP24A1 being expressed in tissue from three donors, CES3 in tissue from two donors and AKR1A4 and UGT2A3 each being detected in only one EpiDerm™ sample.

Most of the 13 genes detected in FTHBS but not in EpiDerm™ tissues were Phase I enzymes (CYP1A1, CYP2A6, CYP2E1, CYP4X1, CYP8B1, PTGIS, aldehyde dehydrogenase (ALDH) 1B1, ALDH3B1 and FMO3). Of these, only CYP1A1, CYP2E1, CYP4X1 and PTGIS were expressed at a signal intensity value greater than 1000 in

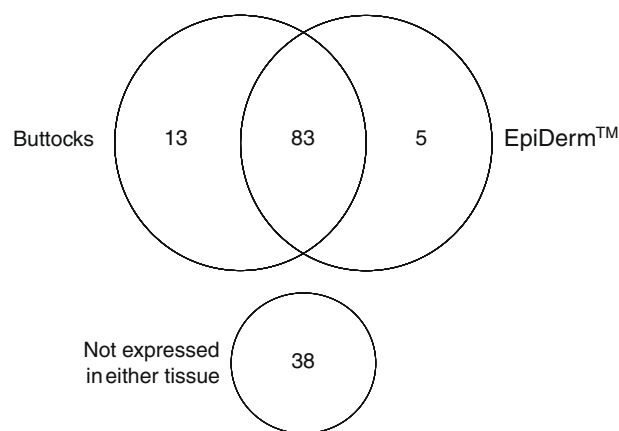


Fig. 1. Venn diagram comparing genes expressed in EpiDerm™ tissues vs full thickness human buttock skin. The numbers of genes identified in Table 1 as being expressed in at least one of the four EpiDerm™ tissues and/or in FTHBS are categorized according to the subsets of samples in which they were detected. Genes whose expression was not detected in any of the tissues tested are shown separately.

Table 2

Overall agreement in present and absent XB metabolism genes between different EpiDerm™ donors and full thickness human buttock skin.

Overall agreement in 139 XB metabolism genes:	Full thickness human buttock skin (%)
EpiDerm™ Donor 254	84
EpiDerm™ Donor 1188	86
EpiDerm™ Donor 219	83
EpiDerm™ Donor 926	84

FTHBS. The relative expression of these four genes in EpiDerm™ tissues in comparison with FTHBS was evaluated further and is discussed below. Though detectable in FTHBS, the expression levels of the other five Phase I genes (CYP2A6, CYP8B1, ALDH1B1, ALDH3B1 and FMO3; signal intensity values of 799, 501, 729, 1057 and 313, respectively, were relatively low. Four Phase II genes (UGT2A1, sulfotransferase (SULT)1A2, cysteine conjugate β -lyase (CCBL) 1 and 3'-phosphoadenosine 5'-phosphosulfate synthase (PAPSS) 2 were found to be expressed in FTHBS but not in EpiDerm™ tissues.

3.2. Affymetrix signal intensity

For a more detailed analysis of gene expression, the Affymetrix gene chip signal intensity of XB metabolizing genes in EpiDerm™ tissues was compared to that in human skin. Heat map analysis (Fig. 2) indicated that the expression levels of genes encoding XB metabolizing enzymes were generally similar in FTHBS and EpiDerm™ tissue from all four donors. Most of the CYP-dependent Phase I genes analyzed were expressed at low to very low levels (signal intensity value ranging from 1 to 1000; shaded blue in Fig. 2a). Other Phase I genes (Fig. 2b) encoding oxidases, reductases and hydrolases were generally expressed at moderate to high levels (signal intensity value greater than 1000; shaded red), consistent with the fact that many of these genes are known to have functions in cellular homeostasis in addition to their roles in XB metabolism. With respect to Phase II genes (Fig. 2c), genes encoding the GST family and its accessory enzymes (glutamate–cysteine ligase, catalytic subunit (GCLC) and glutathione synthetase (GSS)) were expressed at particularly high levels. Certain genes of the UGT and SULT families (UGT1A10 and SULT2B1, together with the gene encoding the sulfate-supplying enzyme PAPSS1) were

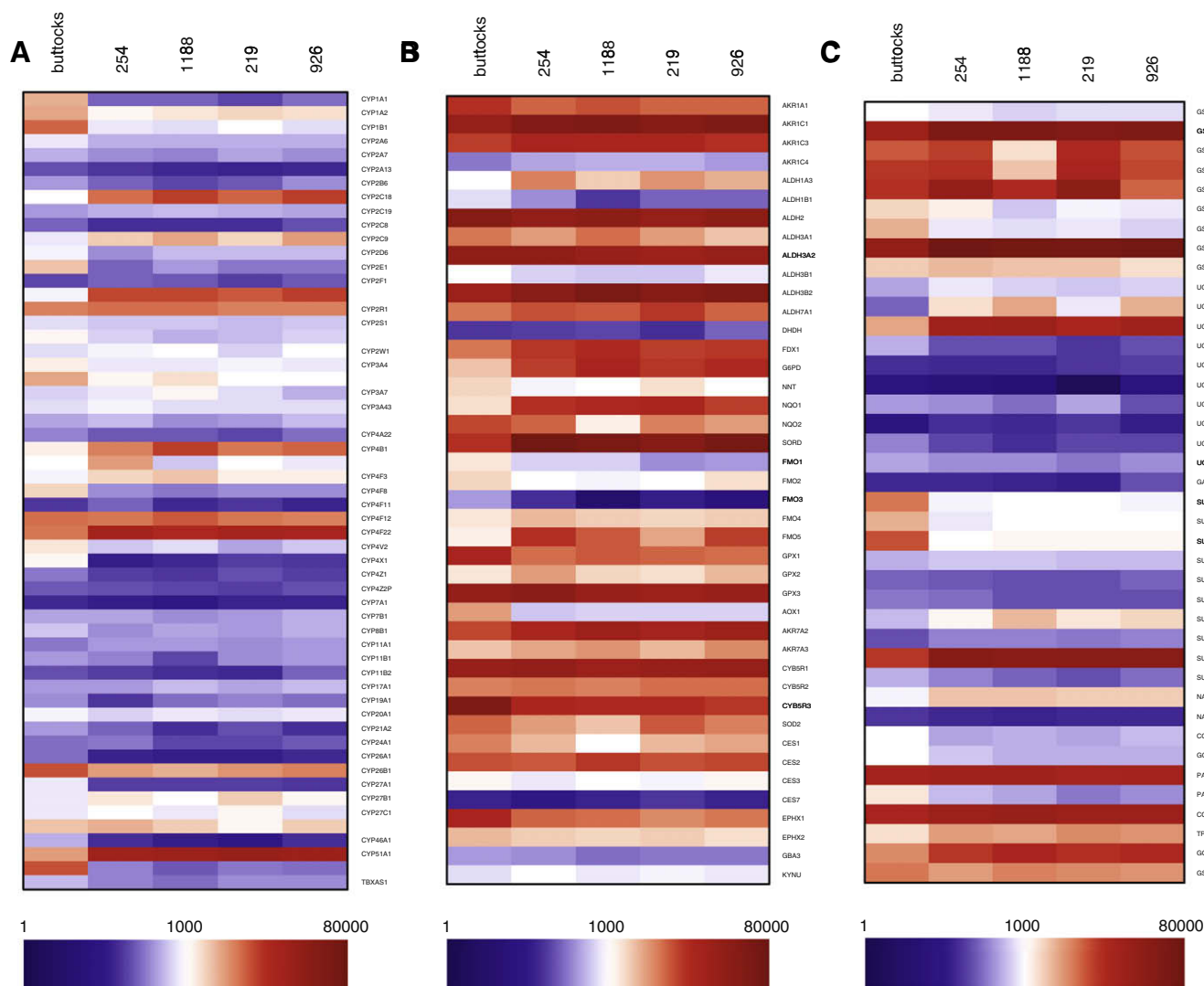


Fig. 2. Heatmap of the xenobiotic metabolizing gene expression level in EpiDerm™ tissues vs full thickness human buttock skin. The signal intensity values are shown on a logarithmic scale for each half of the range of signals covered. Lower signal intensity genes (<1000) are expressed in blue to white color, higher signal intensity genes (>1000) in white to red color. The signal intensity value of 1000 was chosen as the middle point of the scale because it was the closest in magnitude order to the median of the distribution of all signal values. (a) CYP genes (b) non-CYP Phase I genes, and (c) Phase II genes.

Table 3

Over- and under-expression of XB metabolizing genes in EpiDerm™ tissues relative to full thickness human buttock skin.

Gene name	Affymetrix ID	FTHBS signal intensity	Affymetrix signal intensity: fold change relative to FTHBS			
			254	1188	219	926
(a) Phase I cytochrome P450-dependent oxidation enzymes						
Cytochrome P450-dependent oxidases						
CYP1A1	205749_at	2530	−18.1	−16.0	−26.0	−15.1
CYP1A2	207608_x_at	2785	−2.2	−1.9	−1.5	−1.7
CYP1B1	202436_s_at	5095	−6.5	−7.5	−4.7	−7.0
CYP2A6	1494_f_at	799	−1.9	−2.0	−1.9	−2.0
CYP2C9	216025_x_at	781	2.6	3.5	2.3	3.9
CYP2C18	208126_s_at	1102	4.2	6.6	4.8	6.3
CYP2D6	207498_s_at	913	−3.3	−1.9	−2.0	−2.1
CYP2E1	209975_at	2185	−14.5	−7.3	−11.7	−10.6
CYP2J2	205073_at	912	7.4	7.1	5.8	8.1
CYP2R1	227109_at	3896	1.2	1.2	1.0	1.0
CYP2S1	223385_at	708	−1.3	−1.3	−1.5	−1.3
CYP2U1	226393_at	1247	−1.9	−3.0	−2.6	−2.2
CYP2W1	220562_at	691	1.4	1.6	−1.1	1.5
CYP3A5	214235_at	2796	−2.2	−1.8	−2.4	−2.4
CYP3A43	211440_x_at	715	1.3	1.0	1.0	1.0
CYP4A11	1554837_a_at	346	1.3	−1.2	−1.1	1.3
CYP4B1	1555497_a_at	1347	2.8	5.3	3.3	3.8
CYP4F3	206515_at	987	1.8	2.1	1.3	1.4
CYP4F8	210576_at	1765	−6.9	−8.5	−6.8	−6.5
CYP4F12	206539_s_at	4650	−1.1	1.2	−1.1	−1.2
CYP4F22	244692_at	4199	3.1	2.2	2.9	2.7
CYP4V2	226745_at	1464	−2.6	−2.1	−4.1	−2.7
CYP4X1	227702_at	1233	−28.0	−21.2	−13.8	−15.8
CYP7B1	207386_at	343	1.1	−1.2	−1.2	1.2
CYP8B1	232494_at	501	−1.8	−1.4	−1.6	−1.2
CYP20A1	219565_at	993	−1.6	−1.1	−1.4	−1.2
CYP24A1	206504_at	164	1.2	−1.7	−1.6	−1.3
CYP26B1	219825_at	5971	−2.0	−2.4	−1.8	−1.5
CYP27B1	205676_at	760	2.0	1.5	2.5	1.6
CYP27C1	1568868_at	767	1.3	1.1	1.6	1.0
CYP39A1	1553977_a_at	2155	1.2	−1.1	−1.7	−1.1
CYP51A1	216607_s_at	3070	4.9	5.1	6.1	5.0
Prostaglandin I2 (prostagacyclin) synthase						
PTGIS	208131_s_at	5842	−26.7	−46.9	−28.8	−33.6
(b) Other Phase I enzymes						
Oxidases						
Aldehyde dehydrogenase						
AKR1A1	201900_s_at	8301	−1.6	−1.4	−1.6	−1.6
AKR1C1	204151_x_at	20,625	1.8	2.1	1.7	2.0
AKR1C3	209160_at	7245	1.6	1.4	1.4	1.1
AKR1C4	210558_at	188	1.9	2.2	2.3	1.7
ALDH1A3	203180_at	1024	3.7	1.9	3.3	2.4
ALDH1B1	209645_s_at	729	−2.8	−9.8	−5.0	−5.2
ALDH2	201425_at	35,656	−1.6	−1.3	−1.7	−1.4
ALDH3A1	205623_at	4339	−1.5	1.1	−1.5	−2.1
ALDH3A2	202053_s_at	24,656	−1.0	−1.3	−1.4	−1.2
ALDH3B1	211004_s_at	1057	−1.7	−2.0	−2.0	−1.3
ALDH3B2	204942_s_at	16,813	1.9	3.0	2.0	2.6
ALDH7A1	208950_s_at	4324	1.4	1.3	1.8	1.2
Ferredoxin						
FDX1	203647_s_at	4207	1.9	2.3	1.8	1.8
Glucose-6-phosphate dehydrogenase						
G6PD	202275_at	2093	3.6	5.3	3.9	4.6
Nicotinamide nucleotide transhydrogenase						
NNT	202783_at	1771	−2.0	−1.5	−1.1	−1.7
NAD(P)H dehydrogenase, quinone						
NQO1	210519_s_at	1668	5.2	5.7	6.4	4.3
NQO2	203814_s_at	6767	−1.3	−4.8	−1.8	−2.3
Sorbitol dehydrogenase						
SORD	201563_at	8778	7.2	5.4	4.1	6.1
Flavin-containing monooxygenase						
FMO1	205666_at	1441	−2.4	−2.2	−5.5	−4.5
FMO2	228268_at	1793	−1.7	−1.8	−1.6	−1.1
FMO3	40665_at	313	−5.0	−41.7	−7.4	−16.5
FMO4	206263_at	1451	1.6	1.4	1.2	1.3
FMO5	205776_at	1408	6.1	4.0	1.9	5.0
Glutathione peroxidase						
GPX1	200736_s_at	11,596	3.0	−2.1	−2.4	−2.5

Table 3 (continued)

Gene name	Affymetrix ID	FTHBS signal intensity	Affymetrix signal intensity: fold change relative to FTHBS			
			254	1188	219	926
GPX2	202831_at	1504	2.1	1.2	1.1	1.5
GPX3	201348_at	22,148	1.3	−1.1	−1.3	−1.2
Aldehyde oxidase						
AOX1	205083_at	3034	−5.9	−5.0	−5.1	−5.0
Reductases						
Aldo–keto reductase						
AKR7A2	214259_s_at	6824	1.6	2.5	1.8	2.4
AKR7A3	206469_x_at	2142	1.3	1.5	1.1	1.7
Cytochrome b5 reductase						
CYB5R1	202263_at	18,924	1.2	1.0	1.0	1.1
CYB5R2	220230_s_at	3766	1.2	1.0	1.3	1.2
CYB5R3	201885_s_at	43,158	−4.4	−4.6	−4.7	−5.5
Superoxide dismutase						
SOD2	221477_s_at	5186	−1.8	−2.5	1.0	−1.4
Hydrolases						
Carboxylesterase						
CES1	209616_s_at	3983	−1.7	−3.6	−1.7	−1.5
CES2	213509_x_at	5983	−1.1	1.3	1.0	1.1
CES3	220335_x_at	1232	−1.5	−1.2	−1.4	1.0
Epoxide hydrolase						
EPHX1	202017_at	10,961	−2.1	−2.3	−3.1	−2.7
EPHX2	209368_at	2259	−1.2	−1.2	−1.2	−1.4
Glucosidase, beta, acid						
GBA3	219954_s_at	286	1.0	−1.7	−1.4	−1.5
Kynureninase						
KYNU	204385_at	720	1.7	1.1	1.3	1.2
<i>(c) Phase II enzymes</i>						
Transferases						
Glutathione S-transferase						
GSTA1	215766_at	1177	−1.5	−1.8	−1.7	−1.6
GSTA4	202967_at	15,251	2.9	3.0	2.4	2.8
GSTM1	215333_x_at	5518	1.3	−3.5	1.8	1.1
GSTM2	204418_x_at	7539	1.1	−3.7	1.5	−1.1
GSTM3	202554_s_at	8364	2.6	1.2	3.2	−1.7
GSTM4	210912_x_at	1692	−1.3	−3.1	−1.7	−2.2
GSTM5	205752_s_at	2532	−3.0	−3.8	−3.3	−4.1
GSTP1	200824_at	23,088	3.0	2.5	2.4	2.7
GSTZ1	209531_at	2009	1.1	1.0	1.1	−1.3
UDP-glucuronosyltransferase						
UGT1A6	232654_s_at	369	2.1	1.6	1.5	1.8
UGT1A8	221305_s_at	139	11.5	20.3	5.5	18.7
UGT1A10	204532_x_at	2657	5.3	6.0	3.9	5.6
UGT2A1	207958_at	402	−3.6	−3.6	−5.4	−3.8
UGT2A3	219948_x_at	63	−1.2	−1.2	1.2	1.3
Sulfotransferase						
SULT1A1	203615_x_at	4374	−5.0	−4.2	−4.2	−4.8
SULT1A2	207122_x_at	2552	−3.0	−2.5	−2.3	−2.2
SULT1A4	210580_x_at	6023	−5.7	−4.9	−4.9	−4.9
SULT1E1	219934_s_at	452	2.9	5.1	3.4	3.8
SULT2B1	205759_s_at	7873	4.5	3.8	4.0	4.1
N-acetyltransferase						
NAT1	214440_at	869	2.4	2.4	2.2	2.1
Cysteine conjugate β-lyase						
CCBL1	206037_at	1005	−2.9	−2.4	−2.8	−2.1
γ-Glutamyl transferase						
GGT7	226470_at	1059	−1.9	−2.7	−2.5	−2.7
3'-Phosphoadenosine 5'-phosphosulfate synthase						
PAPSS1	209043_at	13,226	1.1	1.1	−1.1	1.0
PAPSS2	203060_s_at	1443	−3.2	−4.1	−6.8	−5.4
Catechol-O-methyltransferase						
COMT	208818_s_at	10,958	1.6	1.7	1.4	1.4
Thiopurine S-methyltransferase						
TPMT	203672_x_at	1613	1.9	1.7	2.1	2.1
Ligases						
Glutamate–cysteine ligase, catalytic subunit						
GCLC	202923_s_at	3512	2.3	3.2	2.5	2.8
Glutathione synthetase						
GSS	211630_s_at	4325	−1.4	−1.1	−1.3	−1.4

Pale grey shading indicates genes expressed at levels at least threefold lower in two or more EpiDerm™ donors than in FTHBS; dark grey shading indicates genes expressed at levels at least threefold higher in two or more EpiDerm™ donors than in FTHBS.

Table 4

Characteristics of genes over- and under-expressed in EpiDerm™ tissues.

Designation	Official identification	Function	Reported expression in human skin	Signal intensity (FTHBS)	Overexpression
<i>(a) Genes exhibiting overexpression in EpiDerm™ tissue</i>					
CYP2C9	Cytochrome P450, family 2, subfamily C, polypeptide 9	Metabolism of various therapeutic agents Goldstein (2001)	Detected in epidermis by immunohistochemistry Enayetallah et al. (2004)	781	2.3–3.9-fold
CYP2C18	Cytochrome P450, family 2, subfamily C, polypeptide 18	Metabolism of various therapeutic agents but of lower clinical relevance than CYP2C9 Goldstein (2001)	Reported to be most abundantly expressed CYP2C mRNA in human epidermis. Subject to exon skipping and formation of circular RNAs Yengi et al. (2003) and Zaphiropoulos (1997, 1999)	1102	4.2–6.3-fold
CYP2J2	Cytochrome P450, family 2, subfamily J, polypeptide 2	Arachidonic acid epoxygenase: generates 20-hydroxyeicosatetraenoic acid	Below limit of detection by immunohistochemistry in epidermis Enayetallah et al. (2004)	912	5.8–8.1-fold
CYP4B1	Cytochrome P450, family 2, subfamily J, polypeptide 2	Metabolizes XBs, including aromatic amines, leading to tissue-specific toxicities Baer and Rettie (2006) . Involved in production of 12-hydroxyeicosanoids in cornea Mastyugin et al. (2004)	Most dramatically upregulated CYP (356-fold) during differentiation of keratinocytes in culture, as demonstrated at mRNA and protein level Du et al. (2006)	1347	2.8–5.3-fold
CYP51A1	Cytochrome P450, family 51, subfamily A, polypeptide 1	Highly specific for 14 α -demethylation of natural 14 α -methylsterols (e.g. lanosterol). Lanosterol 14 α -demethylation is a key reaction in cholesterol biosynthesis Wang et al. (2008)	Not reported	3070	4.9–6.1-fold
ALDH1A3	Aldehyde dehydrogenase 1 family, member A3	Involved in biosynthesis of retinoic acid Trasino et al. (2007) . May be involved in metabolic activation of cosmetic/perfume components which can cause allergic dermatitis Smith et al. (2000)	Family 1 ALDHs are expressed constitutively in human foreskin, breast and abdominal skin. Cheung et al. (1999) . Also in normal buccal keratinocytes Staab et al. (2008)	1024	1.9–3.7-fold
G6PD	Glucose-6-phosphate dehydrogenase	Cytosolic enzyme in the pentose phosphate pathway; helps to maintain cellular NADPH homeostasis	Expressed in keloids and normal skin Sit et al. (1991) . Upregulated in sun exposed skin; overexpressed in Bowen's disease lesions, adjacent uninvolved skin and solar keratoses Pearse and Marks (1978)	2093	3.6–5.3-fold
NQO1	NAD(P)H dehydrogenase, quinone 1	Reductive metabolism of quinones, quinone amines and azo dyes	Expressed in human and murine skin; part of Ah-responsive gene battery Merk et al. (1991) . Detectable in EpiSkin® at levels comparable to <i>ex vivo</i> epidermis, but very low expression in EpiDerm™ and Skinethic Harris et al. (2002c) . May help to protect epidermis against oxidative stress Foppoli et al. (2005)	1668	4.3–6.4-fold
SORD1	Sorbitol dehydrogenase	Member of long chain alcohol dehydrogenase gene family; part of polyol pathway. Converts glucose to fructose during hyperglycaemia. May also play role in osmotic regulation, regulation of cytosolic NADH/NAD ⁺ ratio	Not reported	8778	4.1–7.2-fold
FMO5	Flavin-containing monooxygenase 5	Metabolizes substrates with a soft nucleophilic atom including therapeutic agents, pesticides such as organophosphates/carbamates and dietary components such as trimethylamines	mRNA expressed in adult human breast skin – exhibits interindividual variation. Also in primary cultured keratinocytes («in fresh skin») and HaCaT cells (~fresh skin). In situ hybridization indicates expression in epidermis, sebaceous glands and hair follicles Janmohamed et al. (2001)	1408	1.9–6.1-fold
UGT1A8	UDP-glucuronosyltransferase 1 family, polypeptide A8	Glucuronidation of a wide range of XBs (e.g. primary amines, and natural products), e.g. 4-hydroxycatechol estrogens, 17-hydroxyandrogens, opioids, apigenin, naringenin Cheng et al. (1998)	Not reported	369	5.5–20.3-fold
UGT1A10	UDP-glucuronosyltransferase 1 family, polypeptide A10	Highly homologous with UGT1A8. Glucuronidates planar aromatic compounds, e.g. benzo(a)pyrene-trans-7,8-dihydrodiol Dellinger et al. (2006) . Also has activity towards retinoic acid Cheng et al. (1999)	Not reported	2657	3.9–6.0-fold
SULT1E1	Sulfotransferase family 1E, estrogen-preferring, member 1	Sulfonates estrogens and catecholestrogens with very low km Falany et al. (2006)	Not reported	452	2.9–5.1-fold
SULT2B1	Sulfotransferase family, cytosolic, 2B, member 1	Selective for sulfonation of 3 β -hydroxysteroids, e.g. DHEA, pregnenolone; also sulfonates cholesterol. May play a role in maintenance of epithelial barrier as this is a key role associated with cholesterol sulfate; supports maintenance of granular-stratum corneal junction Falany et al. (2006) and Higashi et al. (2004)	Detected in normal epidermal samples and cultured human keratinocytes during terminal differentiation. Localized to granular layer of epidermis, colocalized with filaggrin (late marker of differentiation) Higashi et al. (2004)	7873	3.8–4.5-fold

(continued on next page)

Table 4 (continued)

Designation	Official identification	Function	Reported expression in human skin	Signal intensity (FTHBS)	Overexpression
<i>(b) Genes exhibiting low level expression in EpiDerm™ tissue</i>					
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	Addition of molecular oxygen across C=C double bonds within polycyclic aromatic hydrocarbons. May lead to metabolic activation of carcinogens	mRNA detected in cultured Langerhans cells, keratinocytes, fibroblasts and melanocytes Saeki et al. (2002) . Present/inducible in <i>ex vivo</i> human foreskin, organ cultures and keratinocytes. Upregulated in cultured human keratinocytes when suspended in semisolid medium. Sadek and Allen-Hoffmann (1994) . Located in basal cell layer of epidermis in non-UVB exposed human skin Katiyar et al. (2000) . Induced by TCDD in cultured keratinocytes but not fibroblasts Akintobi et al. (2007)	2530	15–26-fold
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	Activates procarcinogens and transforms estrogens to genotoxic 4-hydroxyl-catecholestrogens	Consistently most abundant CYP mRNA in human skin samples and cultured skin cells Saeki et al. (2002) , Smith et al. (2003) and Yengi et al. (2003) . Overexpressed in tumors of all types, including skin Murray et al. (1997) . Weak expression localized to non-basal layer epidermal cells of normal skin Katiyar et al. (2000) . Upregulated by UVB irradiation in primary cultured keratinocytes, HaCaT cells Villard et al. (2002) . Expressed constitutively in normal human keratinocytes; expression peaks 6 h after cells put into suspension Akintobi et al. (2007)	5095	4.7–7.5-fold
CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1	Metabolic activation of low molecular weight carcinogens. Along with alcohol and aldehyde dehydrogenases, can activate cosmetic/perfume components which cause allergic dermatitis Smith et al. (2000)	Protein detected in fresh human breast skin Ashcroft et al. (1997) . mRNA in cultured skin cells from six individuals Saeki et al. (2002) and skin from 27 healthy volunteers (<CYP1B1, 2C18) Yengi et al. (2003)	2185	7.3–14.5-fold
CYP4F8	Cytochrome P450, family 4, subfamily F, polypeptide 8	Involved in regulation of eicosanoid activity. Metabolizes PGH1 and PGH2 by ω -hydroxylation. Only known enzyme able to oxidize PGH to 19-OH PGH. Function in human skin may be linked to lipid peroxidation and keratinocyte proliferation Stark et al. (2006)	Detectable by immunohistochemistry in healthy epidermis ($n = 5$). Upregulated in psoriatic lesions compared with adjacent uninvolved tissue Stark et al. (2003)	1765	6.8–8.5-fold
CYP4X1	Cytochrome P450, family 4, subfamily X, polypeptide 1	Able to metabolize anandamide (arachidonyl ethanolamide) to 14,1-epoxyeicosarienoic ethanolamide Stark et al. (2008)	Not reported	1233	13.8–28.0-fold
PTGIS	Prostaglandin I2 (prostacyclin) synthase	Produces prostacyclin (also known as prostaglandin I2) from prostaglandin H2 (PGH2)	Not reported	5842	26.7–46.9-fold
ALDH1B1	Aldehyde dehydrogenase 1 family, member B1	The second enzyme of the major oxidative pathway of alcohol metabolism. Substrates include endogenous lipid peroxidation products (malondialdehyde, hydroxyalkenals) and retinal	Family 1 ALDHs expressed constitutively in human foreskin, breast and abdominal skin. Cheung et al. (1999)	729	2.8–9.8-fold
FMO1	Flavin-containing monooxygenase 1	Broad substrate range Phillips and Shephard (2008) . Metabolizes substrates with a soft nucleophilic atom including therapeutic agents, pesticides and dietary components. N.B. In human liver, FMO1 is a fetal form whereas FMO3 is the major adult isoform. Koukouritaki et al. (2002)	Expressed in adult human breast skin. Not in primary cultured keratinocytes, HaCaT cells. ISH indicates expression in epidermis, sebaceous glands or hair follicles Janmohamed et al. (2001) . Below level of detection by immunoblotting in cultured human keratinocytes Vyas et al. (2006)	1441	2.2–5.5-fold
AOX1	Aldehyde oxidase 1	Broad substrate specificity including N-oxides, nitrosamines, hydroxamic acids, azo dyes, nitro-polycyclic aromatic hydrocarbons and sulfoxides Terao et al. (1998)	Not reported	3034	5.0–5.9-fold
CYB5R3	Cytochrome b5 reductase 3	Thought to have direct role in XB metabolism including reduction of hydroxylamines and amidoximes to parent amines Higashi et al. (2004)	Not reported	43,158	4.4–5.0-fold
GSTM5	Glutathione S-transferase mu 5	Glutathione conjugation	Non-specific GST activity detected in cultured keratinocytes, EpiDerm™ and SkinEthic but much lower in EpiSkin® and fresh epidermis. Not clear how this relates to GSTM5 expression Harris et al. (2002a)	2532	3.0–4.1-fold

Table 4 (continued)

Designation	Official identification	Function	Reported expression in human skin	Signal intensity (FTHBS)	Overexpression
SULT1A1	Sulfotransferase family 1A, phenol-preferring, member 1	May be involved in metabolic activation of polycyclic aromatic hydrocarbons, N-hydroxyaromatic amines, allylic alcohols and heterocyclic amines. Also sulfonates thyroid hormones and estrogens Falany et al. (2006) and Hempel et al. (2007)	Localized to outer root sheath of hair follicle Falany et al. (2006) and Hempel et al. (2007)	4374	4.2–5.0-fold
SULT1A4	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 4	Aryl and phenol sulfotransferase; also has sulfokinase activity	Not reported	6023	4.9–5.7-fold
PAPSS2	3'-Phosphoadenosine 5'-phosphosulfate synthase 2	Supplies phosphoadenosine 5'-phosphosulfate as sulfate donor for SULTs Xu et al. (2000). Part of a sulfation cascade regulated by nuclear receptor PXR Sonoda et al. (2002)	Not reported	1443	3.2–6.8-fold

also expressed at high levels, as were catechol-O-methyltransferase (COMT) and thiopurine S-methyltransferase (TPMT). Moderate to high expression of NAT1 was detected while the level of expression of NAT2 was very low in both human skin and EpiDerm™ tissue as has been reported by other investigators using the Episkin® and SkinEthic models (Eilstein et al., 2009).

In order to compare the over- or under-expression of genes in EpiDerm™ tissue to human skin, the fold difference in expression of 101 genes that were present in either FTHBS or EpiDerm™ (or both) was evaluated. The 38 genes that were absent from both FTHBS and EpiDerm™ were not included in this analysis. As shown in Table 3, each of the four donors was compared with FTHBS, which was selected for this study since the purpose was to determine how well EpiDerm™ represents total human skin, although it is recognized that full thickness skin contains a variety of cell types not present in EpiDerm™ tissues. Thirty genes were expressed at levels at least threefold higher or lower in two or more of the four EpiDerm™ preparations than in FTHBS as marked in Table 3. Importantly, the majority of genes (71) differed by less than threefold in either direction in tissue from two or more of the EpiDerm™ donors compared to FTHBS. This comparison of EpiDerm™ with FTHBS indicated few differences between the two tissue systems, suggesting similarity in overall function and indicating that the

presence of additional cell types in FTHBS did not markedly affect XB gene expression.

Twenty-eight of the genes in Table 3 that had a threefold difference in two or more EpiDerm™ donors are described in detail in Table 4. Further detailed studies at the protein and functional level are needed to fully understand whether these differences result in functional changes between human skin and skin models. Two genes, FMO3 and UGT2A1 are not described further since they had very low signal intensity values <500 in FTHBS and further decreases seen in EpiDerm™ are likely not biologically important. The numbers of genes exhibiting higher and lower expression in EpiDerm™ tissues than in FTHBS were equal (14 higher, 14 lower) and in many cases, elevated expression of one or more members of a particular gene family was accompanied by lower level expression of other members of the same gene family. Five CYPs (CYP2C9, CYP2C18, CYP2J2, CYP4B1) were expressed at higher levels in EpiDerm™ tissue compared to FTHBS while five CYPs had lower expression (CYP1A1, CYP1B1, CYP2E1, CYP4F8, CYP4X1); one ALDH was higher while one was lower; FMO5 was higher while FMO1 was lower and two SULTs were overexpressed while two SULTs (plus PAPSS2) were under-expressed. In addition, two UGTs (UGT1A8 and 1A10) were overexpressed and one GST (GSTM5) was under-expressed. While these are representatives of different

Table 5

Quantitative reverse transcription PCR analysis of higher and lower expression of selected xenobiotic metabolism genes in EpiDerm™ tissues relative to human breast skin.

Gene name	Fold change relative to human breast skin				Affymetrix overexpression
	254	1188	219	926	
CYP2C9	2.7	1.3	3.8	8.4	2.3–3.9-fold
CYP2C18	5.7	5.7	5.9	7.8	4.2–6.3-fold
CYP4B1	1.6	–3.7	2.8	3.0	2.8–5.3-fold
ALDH1A3	58.8	50.8	23.0	101.1	1.9–3.7-fold
NQO1	9.5	12.3	15.6	7.9	4.3–6.4-fold
FMO5	25.3	–1.0	2.1	28.3	1.9–6.1-fold
UGT1A8	91.2	47.4	9.5	101.3	5.5–20.3-fold
UGT1A10	123.7	62.2	8.9	122.2	3.9–6.0-fold
SULT1E1	4.4	4.3	7.3	4.5	2.9–5.1-fold
SULT2B1	23.5	12.3	10.0	15.0	3.8–4.5-fold
Gene name	Fold change relative to human breast skin				Affymetrix under-expression
	254	1188	219	926	
CYP1B1	–3.4	–1.7	–5.8	–3.7	–4.7 to –7.5-fold
CYP2E1	–31.1	–22.8	–12.1	–14.8	–7.3 to –14.5-fold
CYP4F8	–4.3	–17.2	–5.7	–4.0	–6.8 to –8.5-fold
FMO1	–1.2	–1.7	1.7	1.3	–2.2 to –5.5-fold
GSTM5	–14.0	–16.6	–4.5	–7.4	–3.0 to –4.1-fold
SULT1A1	Absent	4.5	3.8	2.9	–4.2 to –5.0-fold
SULT1A4	–2.8	–1.3	1.1	–1.5	–4.9 to –5.7-fold

Note: CYP1A1 and ALDH1B1 were absent in all qRT-PCR samples.

gene families, both are involved in Phase II conjugation of XBs and often metabolize the same substrates.

3.3. Quantitative reverse transcription PCR confirmation of gene expression

qRT-PCR was used to analyse the expression of 17 of the 28 genes identified as being over- or under-expressed in EpiDerm™ tissues (10 overexpressed and seven under-expressed). In these experiments, XB metabolizing gene expression in EpiDerm™ tissue from Donors 254, 1188, 219 and 926 was compared to that in human breast skin. The fold change results observed in qRT-PCR experiments were compared to the fold change results from the Affymetrix analysis (Table 5). CYP1A1 and ALDH1B1 expression as determined by qRT-PCR were absent in both EpiDerm™ (confirming the results of the initial microarray analysis) and breast skin tissues, so these genes were not included in this analysis. In the Affymetrix gene array studies, CYP1A1 and ALDH1B1 were present in FTHBS but not in EpiDerm™ tissues, indicating some variation in human skin expression of these genes.

For the remaining genes, a similar over- and under-expression trend was observed in both qRT-PCR and Affymetrix analysis. All of the 10 genes overexpressed in EpiDerm™ compared to FTHBS in the Affymetrix analysis were also overexpressed in the qRT-PCR analysis compared to human breast skin (Table 5). One exception was observed for CYP4B1 in Donor 1188, the expression of this RNA being decreased compared to breast skin, but increased five-fold relative to FTHBS (Table 3). Of the seven genes found to be under-expressed in the Affymetrix analysis, six were also confirmed as under-expressed by qRT-PCR analysis. The one exception was SULT1A1, which was increased compared to human skin in qRT-PCR but decreased compared to FTHBS in the Affymetrix analysis. SULT1A1 was below the level of detection in the breast skin used in the qRT-PCR analysis and absent in EpiDerm™ tissues from Donor 254 with very low expression being detected in the other donors, indicating that the calculated increase may not be biologically relevant. Overall, the results from qRT-PCR supported the changes observed in the Affymetrix analysis.

3.4. Comparison among individual EpiDerm™ donors

Comparison of the profiles of gene expression in EpiDerm™ tissues from four donors (Table 1) indicated that almost all the genes had similar Affymetrix absent/present calls among the donors. All but 15 (~10%) of the genes examined were either absent in all donors or present in all donors. Likewise, Table 3 shows that the signal intensity values in tissues from different EpiDerm™ donors were very similar. Genes which differed among the donors included glutathione peroxidase 1 (GPX1), GSTM1 and GSTM2. The expression of GPX1 was increased threefold in tissue from Donor 254 compared with FTHBS but decreased by 2.1–2.5-fold in the other three donors (Table 3), while GSTM1 and GSTM2 were expressed at 3.5–3.7-fold lower levels in tissue from Donor 1188 than in FTHBS and lower than in tissues from the other three donors.

3.5. Induction and function

In general the basal expression of CYPs was found to be low in EpiDerm™ tissues (Fig. 2a). In order to determine whether CYP inducibility was retained in EpiDerm™ samples, induction experiments were carried out using the potent CYP1A inducing agent 3-MC. qRT-PCR results for CYP1A1 and CYP1B1 demonstrated significant induction by 3-MC (10 μ M; Fig. 3), indicating that CYP1A1 and CYP1B1 mRNAs were inducible in EpiDerm™ cultures. The EROD activities of control (untreated and vehicle-treated) EpiDerm™ tissues from all four donors were low, but significant

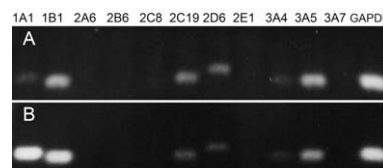


Fig. 3. Representative gel from reverse transcription PCR analysis of CYP genes in EpiDerm™ Donor 254. (a) CYP expression in untreated EpiDerm™ tissue from Donor 254, and (b) CYP expression in EpiDerm™ tissue from Donor 254 tissues after 24 h treatment with 3-MC (10 μ M). Expression was detected by reverse transcription PCR as described.

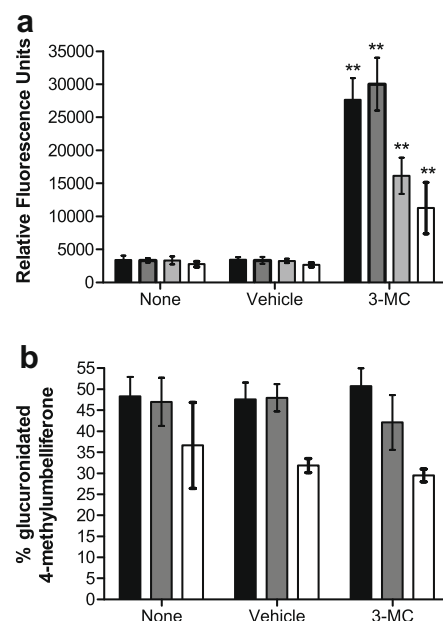


Fig. 4. Xenobiotic metabolizing enzyme activities in EpiDerm™ cultures from different donors. (a) O-deethylation of 7-ethoxyresorufin: EROD activity (mean \pm SD) in EpiDerm™ cultures from different donors was measured by the increase in fluorescence of the CYP1A1/1B1 substrate 7-ethoxyresorufin. The data shown represent experiments repeated at least three times. ■: Donor 254; ■: Donor 1188; ■: Donor 219; □: Donor 926. (b) Glucuronidation of 4-methylumbelliferone: UGT activity (mean \pm SD) in EpiDerm™ cultures from different donors was measured by the decrease in fluorescence of the broad UGT enzyme substrate 4-methylumbelliferone. ■: Donor 254; ■: Donor 1188; □: Donor 926. The data shown represent three independent experiments.

induction of activity was observed following treatment with 3-MC (10 μ M) in tissues from all four donors (Fig. 4a). The extent of induction was greater in EpiDerm™ constructs from donors 254 and 1188 compared to those from the other two donors.

Microarray analysis indicated that UGT1A6 and 1A8 were expressed at low levels in FTHBS but at higher levels in EpiDerm™ tissues (Table 3). EpiDerm™ tissues expressed significant UGT activity, as determined by a 4-methylumbelliferone conjugation assay (Fig. 4b). This activity was not inducible by 3-MC. This, in combination with the high level of gene expression of UGT1A10 (Table 3), supports the conclusion that EpiDerm™ tissues have active detoxification pathways.

The expression of GST genes was relatively high in human skin samples as well as EpiDerm™ tissues (Fig. 2c, Table 3) and high baseline GST activity in EpiDerm™ Donor 254 was verified by measuring GST-dependent conjugation of glutathione with 1-chloro-2,4-nitrobenzene (Table 6). This activity was not induced further by 3-MC.

Table 6

Glutathione S-transferase activity in EpiDerm™ Donor 254.

Sample	GST activity ^a (nmol/min/ml)
Positive control: rat GST enzyme	112.66
Negative control: no enzyme	0.00
EpiDerm™: untreated	307.99
EpiDerm™: 0.1% acetone	275.51
EpiDerm™: 3-MC (10 µM)	262.09

^a The results shown are the means of assays on three EpiDerm™ preparations from Donor 254.

4. Discussion

The results of these studies indicate that the expression of XB metabolism genes in the EpiDerm™ model is very similar to that in human skin as evidenced by excellent agreement in the presence/absence of XB metabolism genes between the two systems, as well as the similarity in signal levels of the genes expressed in both systems. These results support the conclusion that the EpiDerm™ model appears to be a relevant *in vitro* system for the study of cutaneous exposures, metabolism, and toxicity. These studies are a first, essential step in characterizing the metabolic capacity in human skin and the EpiDerm™ model. Since expression of mRNA may not be proportional to functional activity, further detailed studies at the protein and functional level are needed to fully understand metabolic capacity of human skin and skin models. One example of this is our recent report demonstrating NAT1-dependent metabolism of two aromatic amine constituents of hair dyes, *p*-phenylenediamine and *p*-aminophenol, in EpiDerm™ tissue (Hu et al., 2009a). In the present studies, NAT1 had moderate to high expression in EpiDerm™ tissue as well as FTHBS. In metabolism studies, similar N-acetylation of *p*-phenylenediamine and *p*-aminophenol was obtained in EpiDerm™ tissue as has been reported in normal human scalp (Nohynek et al., 2004). These observations support the utility of the EpiDerm™ model for studying effects of aromatic amine hair dye ingredients since NAT1 acetylation in skin is a first step in their metabolism. This is especially relevant to address the 7th Amendment to the EU Cosmetics Directive in March 2009 that prohibits animal testing for skin irritation, corrosion, and genotoxicity testing of cosmetic ingredients, like hair dyes, as of March 2009 and ADME testing of cosmetic ingredients March 2013.

The very similar results obtained using EpiDerm™ tissue from four different donors demonstrate the reproducibility of this model, a key component of an *in vitro* system, especially one constructed from primary human tissue. The concordance between EpiDerm™ tissue and human skin is particularly striking given that the FTHBS tested was from young adult females while all EpiDerm™ tissue preparations are of foreskin origin (i.e. from neonatal males). This suggests commonality in general metabolic capability in human skin from different sites and further supports the relevance of the EpiDerm™ model for studies of cutaneous human exposures.

The genes detected in EpiDerm™ tissues but not in FTHBS were CYP24A1, UGT1A8, UGT2A3, CES3 (carboxylesterase Type 3) and AKR1C4 (3 α -hydroxysteroid dehydrogenase, Type I). UGT1A8 is normally only detected in the gastrointestinal tract (Cheng et al., 1998; Gregory et al., 2003, 2004; Strassburg et al., 1998). UGT2A3 is expressed primarily in the gastrointestinal tract, liver and adipose tissues (Court et al., 2008); however, these authors did not investigate expression in the skin, so the significance of the expression of UGT2A3 in our EpiDerm™ samples is difficult to evaluate.

CYP24A1 is expressed in human skin cells, including the keratinocyte cell line HPK1A-*ras*, where it is able to catalyse the hydroxylation of 1 α -hydroxyvitamin D (Masuda et al., 2006). The

expression of this isoform in EpiDerm™ tissues from 3/4 donors but not in tissue from the fourth donor or in FTHBS may reflect interindividual differences in gene expression, differences in tissue localization (foreskin vs buttocks), developmental regulation (neonate vs adult) and/or sexual dimorphism (male vs female). Little is yet known regarding the normal expression and function of CES3 (Sanghani et al., 2004, 2009), but this isoform appears to have low catalytic activity under a range of assay conditions (Williams et al., 2008) so its expression in EpiDerm™ constructs is unlikely to have a marked effect on the metabolic capacity of the tissue. The 3 α -hydroxysteroid dehydrogenase gene AKR1C4 was expressed in a single EpiDerm™ tissue construct. Since this gene is normally expressed only in the liver (Penning et al., 2000), its expression in 1/4 EpiDerm™ constructs is unlikely to be of biological significance.

Most of the genes detected in FTHBS but not in EpiDerm™ tissues were Phase I enzymes. Of these, CYP2A6, CYP8B1, ALDH1B1, ALDH3B1 and FMO3 were relatively low (signal intensity values of 799, 501, 729, 1057 and 313, respectively); thus their absence in EpiDerm™ tissue is unlikely to lead to biologically significant deficiencies in metabolic capacity compared with human skin. The four Phase II genes (UGT2A1, sulfotransferase (SULT)1A2, cysteine conjugate β -lyase 1 (CCBL1) and 3'-phosphoadenosine 5'-phosphosulfate synthase 2 (PAPSS2) that were found to be expressed in FTHBS but not in EpiDerm™ tissues had only low to moderate expression in FTHBS (signal intensity values in FTHBS of 402, 2552, 1005 and 1443, respectively), and with the exception of cysteine conjugate β -lyase each of them is part of a multigene family other members of which are expressed in EpiDerm™ tissues. It is unlikely, therefore, that deficient expression of these four Phase II enzymes has a marked effect on the XB metabolizing capacity of EpiDerm™ tissues.

The profile of XB metabolizing gene expression in the EpiDerm™ model is overall very similar to that of human skin and the few differences that have been observed may not affect the outcome of generic screening assays. Though constitutive CYP gene expression in EpiDerm™ is generally low, results with chemicals such as cyclophosphamide, which was positive in the RSMN assay (Kaluzhny et al., 2008) indicate that EpiDerm™ constructs are capable of mediating metabolic activation to genotoxic species. Cyclophosphamide is metabolized by CYP2B6 (absent and low signal value in our studies) and CYP3A4 (present but low signal value). Further, some genes are inducible in these models, such as CYP1A1 and CYP1B1 (Figs. 3 and 4). Our results on inducibility of CYP1A1/1B1 are consistent with those of Harris et al. (2002b), who reported low basal activity but responsiveness of 3-MC induction in preparations from 5/6 EpiDerm™ batches compared with 2/5 EpiSkin® batches and 2/6 SkinEthic batches. Despite the similarity in *in vitro* skin models and human skin, it is important to recognize that there may be some changes in the substrate specificity of metabolism and/or the pathways followed by specific XBs that would need to be taken into consideration when undertaking detailed evaluation of the metabolism and potential cutaneous toxicity of a specific compound.

Overall, these data expand our understanding of XB metabolizing capability in the EpiDerm™ model, and are consistent with reports by other investigators (Luu-The et al., 2009; Smith et al., 2003, 2006; Yengi et al., 2003), recently reviewed by Oesch et al. (2007) indicating a generally higher level of expression of detoxifying enzymes and low expression of enzymes involved in metabolic activation in human skin. The results provide further support for the concept that XB metabolizing enzymes expressed in the skin may contribute to the role of skin as a protective barrier by detoxifying XBs following topical exposure. This is an important consideration in the safety assessment of topically applied chemicals.

With respect to recent observations, the genes found to be expressed at low levels in human skin by Duche et al. (2009) (CYP4B1, CYP4F12, CYP26B1, CYP27A1, CYP39A1, CYP51A1, AKR1C1, CES1, CES2, GSTM1, GSTM2, GSTM3, GSTM5, GSTP1, SULT1A1, SULT2B1 and COMT) were detected at similar levels in our studies. These investigators also demonstrated a similar pattern of XB metabolizing gene expression in human skin and the EpiSkin® model (both original and full thickness model versions) using real-time quantitative PCR analysis (Luu-The et al., 2009). Their results were remarkably similar to those reported here in EpiDerm™, indicating low expression of all but two (CYP4B1 and CYP26B1) of the 26 CYP genes they examined, together with higher level expression of non-CYP Phase I and Phase II enzyme-encoding genes (including GSTs, SULT2B1b and COMT).

In conclusion, these and previous results published by our laboratories indicate that the EpiDerm™ model appears to be a valuable, robust *in vitro* tool to help address the need for improved non-animal alternative methods for evaluation of efficacy, metabolism, and toxicity of cutaneous exposures.

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Conflict of interest

Two of the authors (P. Hayden and J. Bolmarcich) are employed by MatTek Corporation, manufacturer of the EpiDerm™ reconstructed human tissue model used in the experiments. The other authors are employed by The Procter and Gamble Company with no conflict of interest to declare.

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