EVALUATION OF A NOVEL MICRONUCLEUS ASSAY USING A HUMAN 3-D SKIN MODEL, EPIDERM™

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Abstract

For many chemicals and products, most notably cosmetics, skin is the highest exposed tissue; however, very few assays are available to directly address potential genotoxicity to this tissue. Although rodent models are being developed to measure micronucleus induction in the skin, European legislation such as the 7th amendment to the Cosmetics Directive precludes the use of in vivo assays for genotoxicity assessments of cosmetic ingredients after 2009. To provide a useful alternative, we are developing an in vitro human skin micronucleus assay using the 3-D EpiDermTM skin model (MatTek Corp, Ashland, MA). Theoretically such a model could approximate the complexities typical of in vivo exposures, e.g. absorption, tissue specificity, metabolism, etc., and at the same time reflect human-specific responses in these parameters. Our standard assay utilizes two 10 ul doses of test material applied to the surface of the EpiDermTM tissue 24 hours apart, with harvest 24 hours after the last dose. Using this procedure we show dose related increases in both cytotoxicity and micronuclei induction for several model genotoxins including mitomycin C (maximum micronucleus response [MMR] ~8% at 0.6 ug total dose), vinblastine sulfate (MMR ~4% at 0.01 ug total dose), methylmethane sulfonate (MMR ~0.6% at 20 ug total dose), and N-methyl-N'-nitro-N-nitrosoguanidine (MMR ~0.7% at 40 ug total dose). The background frequency of micronuclei is low at <0.1% (N=20), with a positive control of 3 ug/mL or 6 ug/mL MMC resulting in 1.2% micronucleated cells/binucleated cell (N=7) and 2.3% micronucleated cells/binucleated cell (N=8), respectively. We have initiated studies to investigate whether the model will respond to genotoxins requiring metabolic activation and find that EpiDermTM cultures from different donors express numerous genes associated with xenobiotic metabolism that are also found in normal human skin. This novel assay system appears to hold excellent promise as a human “in vivo-like” genotoxicity model.

Introduction

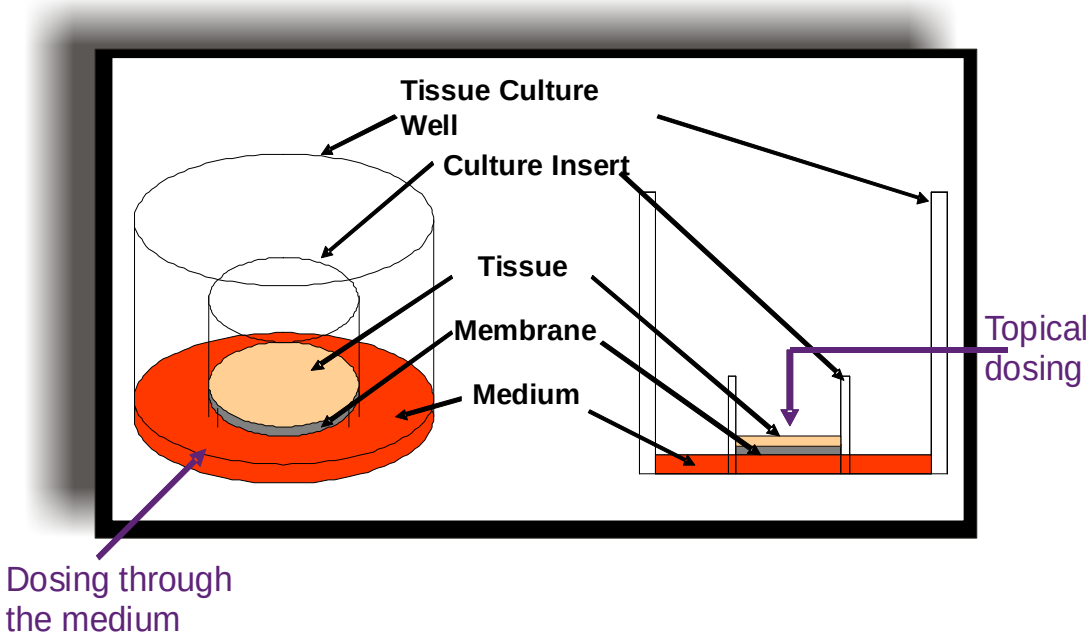
The *in vitro* micronucleus assay has been well established for use in detecting potentially clastogenic and aneugenic chemicals (1,2). Its acceptance has been especially helped by the development of the cytokinesis-block methodology which allows the identification of cells which have undergone one nuclear division, and highlights differences in cell division kinetics due to cytotoxicity, growth conditions or cell origin. Chemicals that are active in the in vitro micronucleus assay are evaluated in vivo (generally using the hematopoietic system as the target [3]) to clarify the in vitro results. These in vivo systems have been useful in assessing more accurately the actual risk from a chemical since the traditional in vitro genotoxicity screens are known to have low specificity, i.e. they generate many false positives (4). It would be very useful if non-animal models were developed which could perform with the necessary high specificity.
Recently an interest has developed in utilizing a tissue, skin, that is often the tissue with highest exposure to natural substances and consumer products. However, the in vivo micronucleus assay in skin utilizes rats or mice (5-8) and may not be completely reflective of what would occur in humans. Further, the use of animals poses a difficulty since new regulations in Europe such as the 7th Amendment to the Cosmetic Directive and the REACH program, will make it very difficult, and in some cases impossible, to use animal models in safety assessments.

Therefore we have investigated human three-dimensional skin constructs as a target system for micronucleus studies (9-10). Theoretically such models could reflect the complexities typical of in vivo exposures, e.g. absorption, tissue specificity, metabolism, etc., as well as human-specific responses in these parameters. We have used a commercially available skin model, EpiDermTM (MatTek Corp., Ashland, MA), to begin our studies with a set of model genotoxicants, mitomycin C (MMC), vinblastine sulfate (VB), methylmethanesulphonate (MMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Importantly, we have also used rodent skin non-carcinogens to begin investigation of the specificity of this new non-animal genotoxicity model.

Assay Development

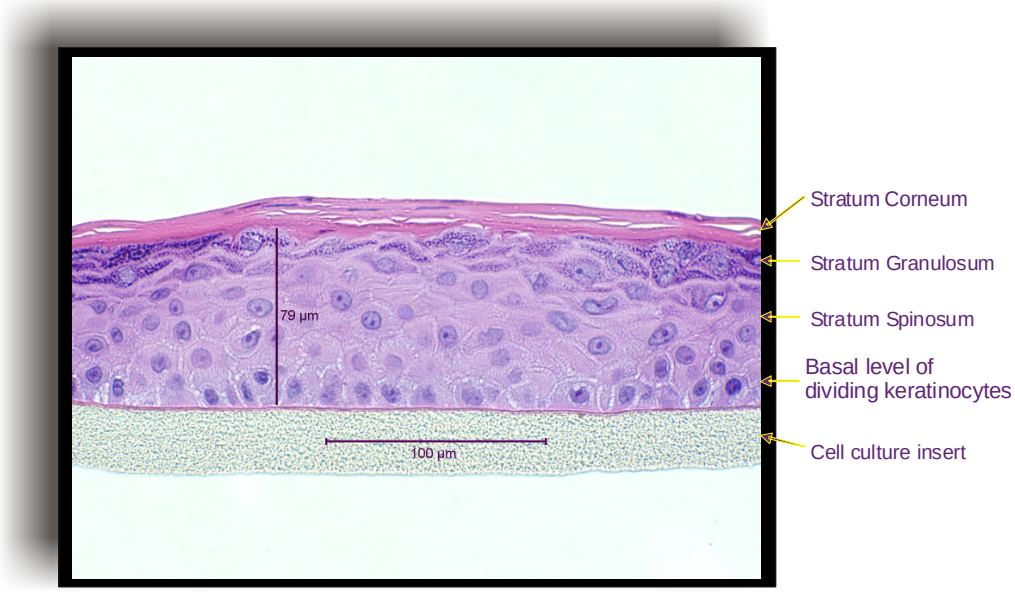
EpiDerm™ is a commercially available skin model (0.64 cm2) supplied in a 24-well kit. Histology shows many similarities to normal human skin. For the MN assay, tissues are grown in cytochalasin B to inhibit cytokinesis and the dividing cells (mostly basal cells and stratum spinosum) are isolated and scored for MN in binucleated (once divided) cells.Test materials can be applied either through the medium or topically (preferred).

Figure 1.



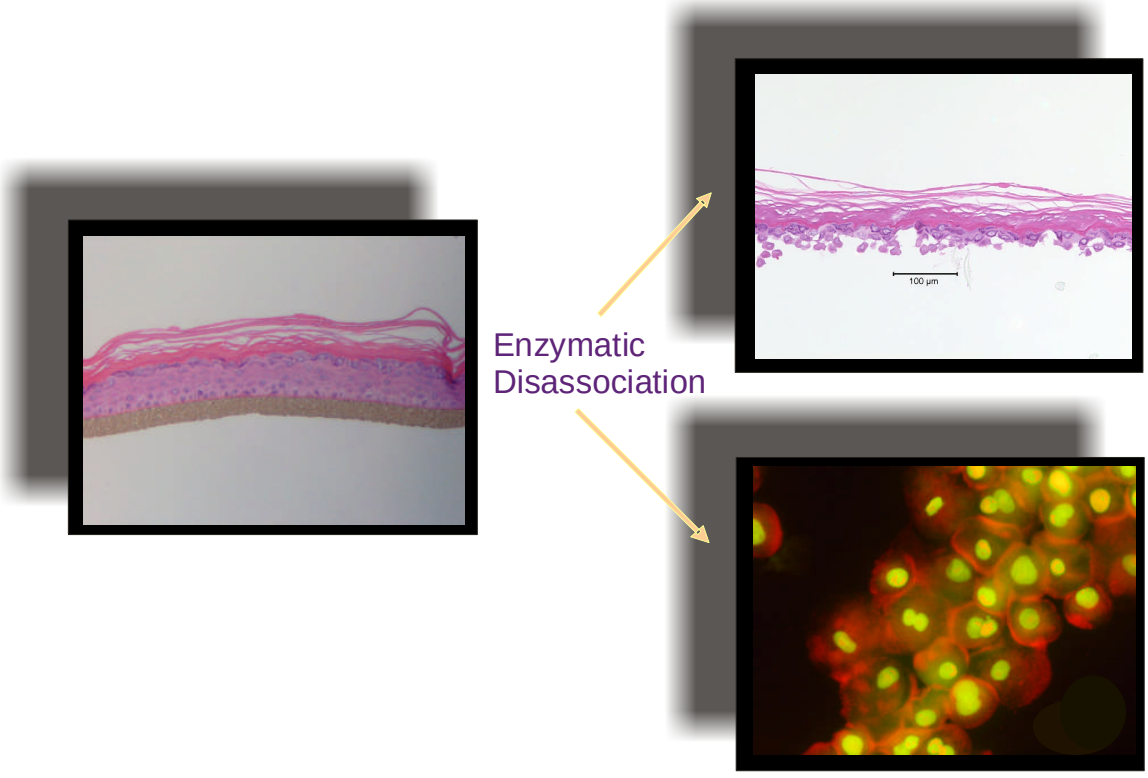
A schematic representation of the EpiDermTM tissue as used in these experiments. The air-lifted tissue sits on a collagen-coated membrane with its stratum corneum exposed to the atmosphere. It is fed by medium diffusing up through the membrane. Diagram courtesy of MatTek Corp

Figure 2.



Cross section of an EpiDermTM tissue on arrival at the laboratory after overnight shipping. H&E. Photo courtesy of J. Harbell, IIVS.

Figure 3.



EpiDermTM tissue is enzymatically disassociated to yield a single cell suspension (mostly basal and spinous cells), and a pad of stratum corneum and stratum granulosum cells (discarded). AO - stained cells (many binucleated) photographed with fluorescence illumination.

Materials and Methods

Tissue Constructs

EpiDerm™ tissue (EPI-200), MatTek Corporation (Ashland, MA) is a multilayered, differentiated, tissue with basal, spinous, granular and cornified layers (see Fig. 2) resembling the normal human epidermis. The tissue is constructed from normal epidermal keratinocytes (foreskin-derived) cultured on collagen-coated, 9 mm ID cell culture inserts (e.g. Millicell CM or Nunc). Differentiation is induced by air-lifting the growing cultures so that the cell insert is at the surface of the medium and the apical surface of the tissue is exposed to the atmosphere (Fig. 1). Tissues are shipped to the user cold overnight in 24 well plates. Tissues in NMM media (MatTek Corp.) maintain good morphology for experimental duration of up to 4 days after receipt.

Treatment Conditions

Tissues were treated with test chemical either topically or through the medium (Fig. 1). For topical dosing, the test material was prepared in the appropriate solvent (either EtOH or acetone), and two, 10ul applications were applied 24h apart, directly to the surface of the EpiDermTM tissue with a micropipette. Cells were harvested 24h later. Statistically significant increases in micronucleated cells are shown by * where $p<0.05$ was considered significant (Fisher's Exact test).

Cell harvesting & slide preparation procedure

Tissues were harvested ~24 hours following the final dosing in the following fashion:

1. Tissues were washed with DPBS, followed by EDTA exposure, and then exposed to trypsin-EDTA at RT ~15 min.
2. Tissues were separated from the membrane and again exposed to trypsin-EDTA for ~5 min. Fig. 3 illustratrates the results of the keratinocyte isolation procedure.
3. Trypsin-EDTA neutralizing solution was added to the cell suspension before centrifuging at 100Xg for 5 minutes.
4. After centrifugation, 1 ml of warm (~37oC) KCl was slowly added to the cells followed by 3 ml of cold MeOH/acetic acid (3:1).
5. The cell suspension was recentrifuged, the medium removed and 4 ml of MeOH/acetic acid were added.
6. The cell suspension was again centrifuged and the cells were gently placed on the slide immediately, or more generally after several days of refrigeration.
7. Slides were stained in acridine orange (AO) solution for 2 to 3 minutes, rinsed, and then scored using a fluorescent microscope.

RT-PCR

Total RNA was isolated from the human skin (unutilized surgical, breast) and EpiDerm cultures with RNAqueous, Ambion. RT-PCR of P450 messages was performed with a commercially available kit containing optimized primers for 11 human P450 isozymes and a housekeeping gene (Human P450 Gene Family I MultiGene-12™ RT-PCR Profiling Kit: PH-027B and ReactionReady™ HotStart "Sweet" PCR master mix, SuperArray Bioscience, Frederick, MD).

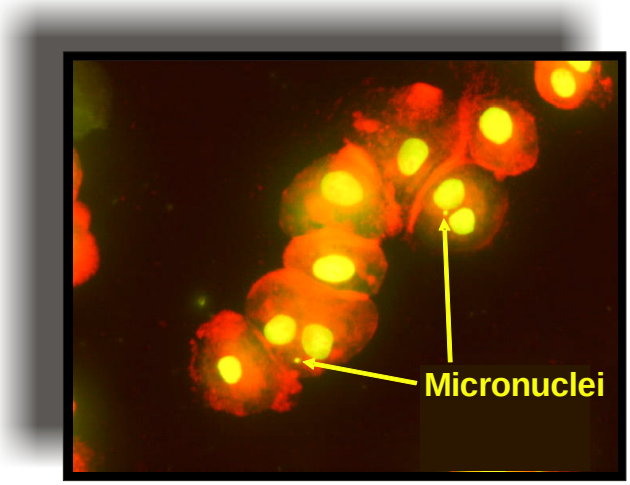
Affymetrix Gene Expression Analysis

Total RNA was isolated from the human skin (10 samples from young women, sun-protected skin) and EpiDerm cultures (3 samples from each of two different donors). Ten normal human skin samples and 3 EpiDerm samples were analyzed by Affymetrix U133A GeneChips. 3 Procter & Gamble (PG) EpiDerm samples were analyzed by Affymetrix HuGeneFL GeneChips. Genes involved in xenobiotic metabolism were compared among three datasets. Affymetrix Present (P) and Absent (A) calls from analyzed chips were used as a basis of our data comparisons.

Assay Procedures

We have completed initial studies to investigate the ability of the new assay to detect traditionally positive genotoxic materials and have found that mitomycin C, vinblastine, methylmethanesulphonate, and N-methyl-N'-nitro-N-nitrosguanadine are positive in the assay. With any new assay it is very important to determine specificity as well as sensitivity; therefore we have begun investigating its performance with chemicals found to be negative in an in vivo mouse skin MN assay. Our new assay has shown no statistically significant increases of MN with 3 materials that are rodent skin non-carcinogens and are also negative in the in vivo skin micronucleus assay. Negative control tissues have low (~0.1%) background MN frequencies.

Representative Micronuclei



Standard Treatment Protocol

T -24h
Tissues arrive, fed with NMM, incubated at 37°C

T 0h
Tissues refed with NMM & Cyt-B, dosed with test chemical topically or in medium.

T +24h
Tissues refed with NMM & Cyt-B, dosed with test chemical topically or in medium.

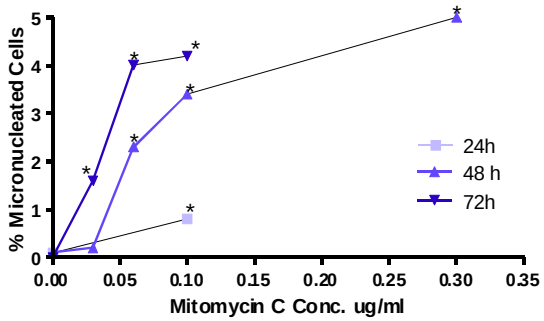
T +48h
Tissues harvested & cells plated & stained; optional histology. Redosed in certain protocols.

T +72h
Tissues harvested & cells plated & stained in certain protocols; optional histology.

Exposure Through the Medium

Figure 4.

Justification for 48h (2 chemical doses) as treatment period



Dose responses for EpiDerm treated 1X, 2X or 3X (24h apart) with 10ul of the indicated MMC concentration.

Control MN Frequency

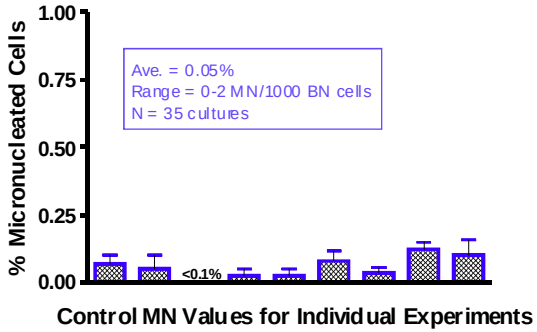
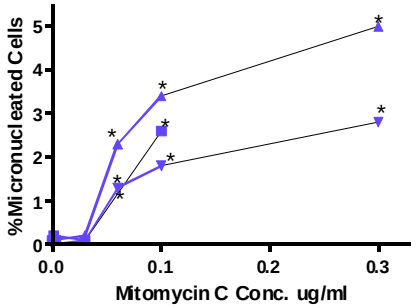


Figure 5.

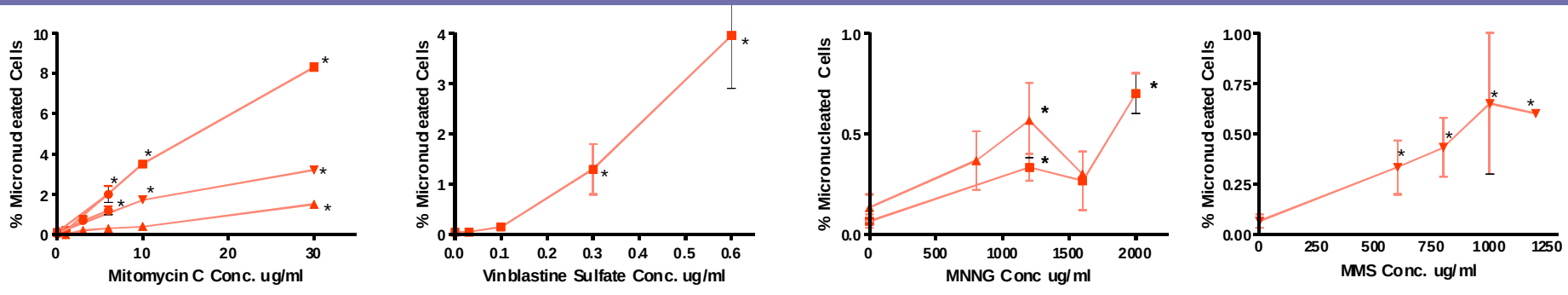
Reproducibility of MMC activity



Dose response for 3 individual experiments using 2X dosing 24 h apart with 10ul of the MMC concentration.

Topical Exposure

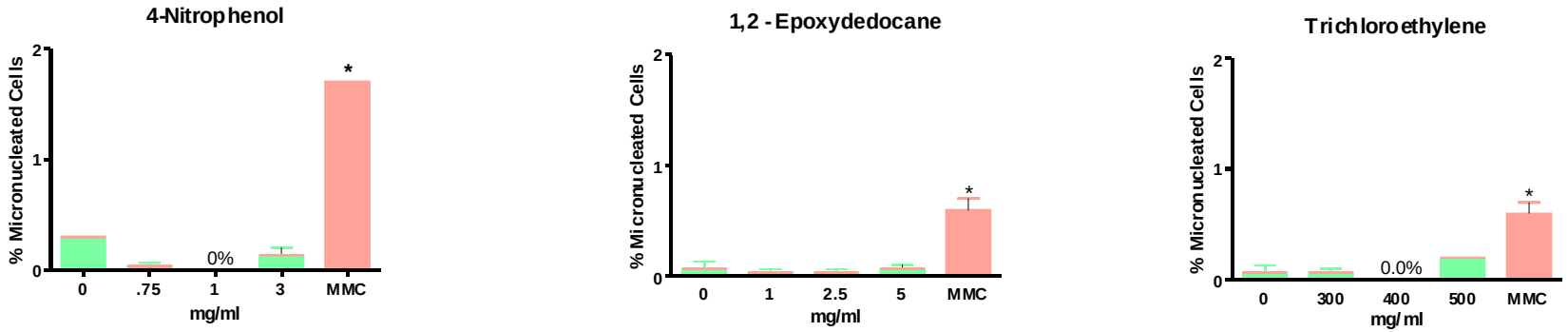
Figure 6. Response of Four Model Genotoxins



Dose responses for model genotoxins dosed twice (24h apart) with 10ul of the indicated concentration and harvested 24h after the last application.

Response of Three Rodent Skin Non-Carcinogens Which Are Also Negative in In Vivo Skin MN

Figure 7.



Lack of MN response for rodent skin non-carcinogens dosed as above. The results with a concomitant ly tested positive control of 3ug/ml MMC is indicated. All have been tested to toxic levels.

Xenobiotic Metabolizing Genes

Figure 8. Expression Patterns

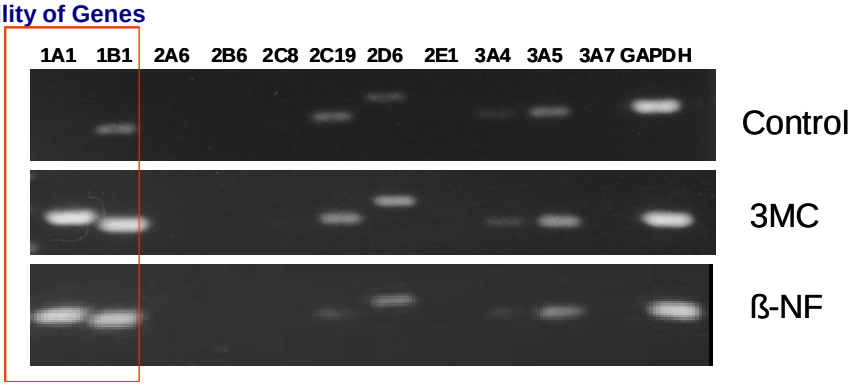
Gene Symbol	Normal Human Skin	MatTeK EpiDerm	PG EpiDerm	Gene Symbol	Normal Human Skin	MatTeK EpiDerm	PG EpiDerm
cytochrome P450				***epoxide hydrolase***			
CYP1A1	P	A*	A	EPHX1	P	P	A
CYP1A2	P	P	P	EPHX2	P	A	P
CYP1B1	P*	A (P)*	A	***flavin containing monooxygenase***			
CYP2A6	P	P	P	FMO1	P	A	A
CYP2B6	A*	A*		FMO2	P	P	A
CYP2C8	A*	A*		FMO3	P	A	A
CYP2C9	P	P	P	FMO4	P	P	A
CYP2C18	P	P	P	FMO5	P	P	P
CYP2C19	P	P*	P*	***glutathione peroxidase***			
CYP2D6	P*	A(P)*		GPX3	P	P	P
CYP2E1	P	A	A	GPX4	P	P	P
CYP2F1	A	A	A	***glutathione S-transferase***			
CYP2J2	P	P	P	GSTM2	P	P	A
CYP3A4	P*	A(P)*	A	GSTM3	P	P	A
CYP3A5	P*	P*		GSTM5	P	P	A
CYP3A7	A*	A*	A	GSTO1	P	P	P
CYP4B1	P	P	P	GSTP1	P	P	P
CYP4F3	P	P	P	GSTT2	P	P	P
CYP11B1	P	P	A	***P450 (cytochrome) oxidoreductase***			
CYP17A1	A	A	A	POR	P	P	P
CYP24A1	A	A	A	***UDP glycosyltransferase***			
CYP51A1	P	P	P	UGT1A10	P	P	P
N-acetyltransferase				UGT2B15	A	A	A
NAT1	P	P	A	UGT2B4	A	A	A
NAT2	A	A	A	UGT8	A	A	A

*: Genes confirmed by RT-PCR.

? : Blue indicates genes detected as present in both normal Human skin and EpiDerm samples.

Expression pattern of selected xenobiotic metabolizing genes expressed in preparations of EpiDermTM tissue from different donors and compared to that of normal human skin.

Figure 9. Inducibility of Genes



Inducible with 3MC: 1A1, 1B1, 2C19, 2D6, 3A4, 3A5

Inducible with b-NF: 1A1, 1B1,2D6,3A5

Comparison of constitutive levels of selected CYP genes with levels after induction with 10 uM 3-MC or 20 uM â-NF for 24 hours.

Conclusions

The micronucleus assay using a human 3-D skin model has numerous features that make it attractive as a future addition to a standard genetic toxicology battery, especially for chemicals and products subject to the EU's 7th Amendment to the Cosmetics Directive.

- EpiDerm™ tissue has a low and reproducible background frequency of micronucleated cells.
- Test materials can be applied to the surface of the tissue allowing allowing comparisons with in vivo dermal studies.
- The tissue is responsive to cytochalasin B which facilitates cytotoxicity analysis and MN scoring.
- Cell replication is adequate for MN scoring with 35 - 55% of cells having divided during the exposure period.
- Positive and negative materials tested to date have been correctly identified.
- Gene expression data show that EpiDerm™ cultures from different donors express numerous genes that are associated with xenobiotic metabolism and that are found in normal human skin. Several of these genes are inducible in EpiDerm.

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