Published in final edited form as:

Toxicol In Vitro. 2008 August ; 22(5): 1273–1284.

Evaluation of In Vitro Cytotoxicity and Paracellular Permeability of Intact Monolayers with Mouse Embryonic Stem Cells

Anthony R. Calabro, Roula Konsoula, and Frank A. Barile

St. John's University, College of Pharmacy and Allied Health Professions, Department of Pharmaceutical Sciences, Toxicology Division, Queens, New York

Abstract

Mouse embryonic stem (mES) cells were induced to form intact monolayers in cell culture inserts, using combinations of extracellular matrix (ECM) components and growth factors (GFs). Progressive formation of intact monolayers was monitored using transepithelial electrical resistance (TEER) and passage of paracellular permeability (PP) markers. The mES cells were initially inoculated on inactivated mouse embryonic fibroblasts (MEFs) plus leukemia inhibitory factor (LIF). At 75% confluence, cells were passaged in the absence of MEF and LIF to stimulate formation of rounded multicellular aggregates (MA). After 4 days, cultures containing MA were transferred to culture inserts coated with ECM components only, and grown in the presence of selected individual GFs. An additional 10 to 14 days revealed confluent monolayers with TEER values of 500-700 ohms-cm² (Ω -cm²). Monolayers grown on inserts coated with ECM components, such as fibronectin or collagen-IV, in the presence of epidermal growth factor or keratinocyte growth factor in the medium, yielded the highest TEER measurements when compared to cultures grown without GFs or ECM. Acute cytotoxicity (AC) studies with confluent monolayers of mES cells in 96-well plates indicated that there is a high correlation ($R^2=0.91$) between cell viability and TEER for 24-h exposure time. Also, decrease in TEER is inversely proportional with increase in PP of markers. In comparison to standardized Registry of Cytotoxicity (RC) data and TEER measurements, MTT IC₅₀ values for mES cells are lower. Thus, at equivalent concentrations for the same chemicals, cell viability decreases before the integrity of the monolayer is compromised. This system represents a novel approach for the manipulation of mES cells toward specific intact monolayers, as an *in vitro* model for biological monolayer formation, and most importantly, for applications to cytotoxicity testing.

Keywords

in vitro cytotoxicity; mouse embryonic stem cells; paracellular permeability; transepithelial electrical resistance; MTT assay

1. Introduction

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) recommended the development and

Address Correspondence to: Frank A. Barile, Ph.D., Associate Professor, Department of Pharmaceutical Sciences, St. John's University College of Pharmacy, 8000 Utopia Parkway, Queens, NY 11439, barilef@stjohns.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

submission of novel *in vitro* methods as simple predictive models for human acute cytotoxicity (AC). Optimization of these systems should also yield models that mimic gastrointestinal absorption, dermal toxicity, and acute lethality *in vivo*, as well as predict rodent LD₅₀ values (ICCVAM publications 01-4499 and 01-4500, 2001). To address this problem, several investigators have recommended the ZEBET¹ approach as a strategy to reduce the number of animals required for acute oral toxicity testing (Halle *et al.*, 2000; Spielmann *et al.*, 1999). The method uses *in vitro* cytotoxicity data to determine the starting dose for *in vivo* testing, by calculating the standard regression between mean IC₅₀ values and corresponding acute oral LD₅₀ data (Registry of Cytotoxicity, RC; ICCVAM publication 01-4499, 2001). The regression could then be used to estimate the LD₅₀ value of a new compound as the *in vivo* starting dose of a study. The application of this technique, however, is limited by the lack of information on *in vitro* models for gastrointestinal, dermal and blood-brain barrier passage and biotransformation (Curren *et al.*, 1998). In fact, monolayers of intestinal and colonic epithelial cells have been used as cell culture models for detecting transepithelial transport of drugs, paracellular permeability (PP) and other intestinal responses to xenobiotics (Carriere *et al.*, 2001). The effect of chemicals on the relationship between PP and AC testing in an *in vitro* model, however, is not well established.

We recently used Caco-2 monolayers (Konsoula and Barile, 2005) and IEC-18 cells (Konsoula and Barile, 2007) as *in vitro* models for comparing PP with AC of 20 chemicals. Caco-2, an immortal cell line originating from human colon, demonstrate transepithelial electrical resistance (TEER) values that are relatively higher than IEC-18 monolayers cultured from the small intestine. The results from these studies revealed that finite IEC-18 cells grown on cell culture inserts were sensitive to toxic insult as immortal Caco-2 cells, and cell viability decreased before membrane integrity was compromised. IEC-18 cells, however, registered significantly lower absolute TEER values than Caco-2 cells. The lower resistance, as well as the higher transport of chemicals in IEC-18 cells in culture, is analogous to the lower resistance of epithelial barriers within the small intestine (He *et al.*, 1998). The diminished expression of junctional proteins (ZO-1, occludin, e-cadherin) by IEC-18 suggests that junctional complexes are less tightly organized than in Caco-2 (Quaroni and Hochman, 1996). These features also explain the higher TEER resistance of Caco-2 cells and prompt the need to develop uniform, predictable *in vitro* models using diploid cells for measuring AC and PP.

Mouse embryonic stem (mES) cells are derived from pluripotent cells of the early mammalian embryo and are capable of unlimited, undifferentiated proliferation *in vitro* (Evans and Kaufman, 1981). In chimeras with intact embryos, culturing of mES cells provide a powerful approach for introducing specific genetic changes into the mouse cell line (Bradley *et al.*, 1984). Pluripotency allows for the ability of the cells to differentiate to the three embryonic germ layers. However, there are few reports demonstrating the differentiation of any ES cells to epidermal or epithelial structures. For instance, Yamada *et al.* (2002) describe the differentiation of mES cells into a functional gut-like organ *in vitro* that exhibits morphological and physiological properties characteristic of the gastrointestinal tract. Kuwahara *et al.* (2004) report that mES cells undergo *in vitro* organogenesis by forming contracting gut-like organs from embryoid bodies (EBs). These structures are surrounded by epithelium, lamina propria, and muscularis. Ishikawa *et al.* (2004) further characterize interstitial cells of Cajal and smooth muscle cells arising from EBs derived from mES cells. All of these studies were prompted by the developmental understanding that *in vivo* epithelium undergoes continuous renewal by multipotent stem cells, which remain anchored to the crypt base. Thus a single embryonic stem cell has potential to migrate and commit to all of the epithelial lineages (Kirkland and Henderson, 2001).

¹Center for Documentation and Evaluation of Alternative Methods to Animal Experiments, Germany.

Several growth factors, particularly IL-3, (Wiles and Keller, 1991), retinoic acid (Bain *et al.*, 1995), and TGF β 1 (Rohwedel, 1994), have been shown to direct linear specific differentiation of mouse stem cells. Epidermal growth factor (EGF), and the related EGF family member, amphiregulin (AR), are mitogenic polypeptides that induce differentiation into ectoderm and mesoderm (Gritti *et al.*, 1999). Keratinocyte growth factor (Kgf) is an epithelial cell-specific mitogen responsible for normal proliferation and differentiation of epithelial cells (Visco *et al.*, 2004). In most cases, the growth factors were applied to aggregates of ES cells after removal of leukemia inhibitory factor (LIF), a cytokine that inhibits differentiation. In the absence of LIF, mES cells create intracellular contacts and initiate signaling and spontaneous differentiation (Furue *et al.*, 2005). Keller *et al.* (2004) describe the induction of epithelial- or epidermal-specific gene expression and differentiation using a combination of GFs plus extracellular matrix (ECM) components in human ES cells. Based on these reports, we explored the possibility that mES cells could be stimulated to differentiate and form confluent monolayers with high transmonolayer resistance when grown on porous inserts coated with ECM substrata. Moreover, the inclusion of mitogenic GFs in the media, in the absence of MEF layers or LIF, could further promote differentiation.

Thus, two objectives directed our experimental plan. First, we systematically designed a series of experiments aimed at determining which combination of GFs and ECM coatings would guide mES cells toward the formation of intact cultured monolayers, as determined by TEER measurement and passage of PP markers. Second, we used the culture model to develop an *in vitro* system for measurement of AC and PP. The contention is that the mES model improves the predictive ability of *in vitro* acute toxicity testing assays for *in vitro/in vivo* correlations over Caco-2 cells because the latter are a differentiated immortal cell line with higher TEER values and greater resistance to chemical insults than mortal continuous cells. And, although IEC-18 cells are mortal diploid intestinal mouse cells, they do not form intact monolayers with sufficient TEER registry (150-200 ohms•cm²). Finally, our mES cell model follows on the successful development of prediction models using cultures of a pluripotent mouse embryonic stem cell line (embryonic stem cell test) for embryotoxicity testing (Genschow *et al.*, 2000). Thus, the mES cells can be manipulated to form specific intact monolayers with critical tight junction (TJ) formation with significant TEER values, and can be applied to the development of AC testing models.

2. Materials and methods

2.1. Cell culture and chemicals

Cell culture liquid and powder media, and cell culture grade chemicals and supplements were obtained from Invitrogen Corp. (Carlsbad, CA, USA). Biocoat[®] cell culture inserts, Falcon[®] tissue culture multiwell plates, flasks, and other sterile disposable supplies were obtained from Becton Dickinson Labware (Bedford, MA, USA). All other test chemicals, including those listed in Table 1, were obtained from Sigma-Aldrich (Sigma Life Sciences, St. Louis, MO, USA; Aldrich Chemical Co., Inc., Allentown, PA, USA). Chemicals used in the studies were suggested by the Registry of Cytotoxicity (RC; Halle, 2003); they were selected based on the verification of the data set (RC-II), and for their validity in establishing a regression model between oral LD₅₀ and IC₅₀ values from a single mammalian cell line (ICCVAM publication 01-4500, 2001).

Mouse ES cells were propagated in culture with and without mitomycin-C treated mouse embryonic fibroblast layers [MEFs, 3T3 Swiss mouse fibroblasts, CCL-92; American Type Culture Collection (ATCC), Rockville, MD, USA]. MEFs were seeded at 10⁴ cells/cm² in T-75 culture flasks and grown in Dulbecco's modified Eagle's medium supplemented with 10% calf bovine serum (DMEM-10), 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 4 mM glutamine, and 5% antibiotic/antimycotic solution, in an atmosphere of 5% CO₂. When monolayers were

70% confluent, cells were treated with 10 µg/ml mitomycin-C (mito-C) in DMEM-10 for 2-hr at 37°C, after which the plates were washed and passaged with trypsin/EDTA. Pellets were replated at 1:3 dilution in T-75 flasks and allowed to attach overnight.

Mouse embryonic stem cells (mES cells, ES-D3, CRL-1934, ATCC) are derived from blastocysts of a 129S2/SvPas mouse. All studies were performed with cells at passage numbers 10 to 30². In our laboratory, mES cells were maintained in the undifferentiated state by frequent subculture (every 2 days) on confluent mito-C treated MEFs, and in DMEM containing 2 mM L-alanine-L-glutamine and 1000 U/ml leukemia inhibitory factor (LIF; recombinant human LIF, Millipore Corp., Temecula, CA, USA). The medium was adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 0.1 mM 2-mercaptoethanol, 0.5% non-essential amino acids (NEAA), and 15% fetal bovine serum (ES-DMEM). Medium change was performed every day. Morphology of (a) mES cells in the presence of feeder layers, and (b) multicellular aggregates (MA) and differentiated cells, in the absence of feeder layers, was documented using phase contrast microscopy with the Leitz Labovet FS inverted phase contrast microscope equipped with the SPOT[®] Insight 2.0 megapixel microscope camera, and processed with the SPOT[®] advanced photographic software [although the formation of MA in our cultures resembles embryoid bodies, the former are rounded aggregates of cells that appear in monolayer cultures; thus the term EB is restricted to spherical aggregates that appear after 4 to 5 days of growth in the absence of LIF or MEF in suspension culture; see Figures 1 & 2].

2.2. Assay procedures

2.2.1. Propagation of mES cells on culture inserts—Prior to experiments, ES-D3 cells were maintained and propagated in the undifferentiated state (Barile, 2007). To induce differentiation, LIF was removed from the media. Subconfluent mES cells (along with some residual inactivated MEFs) were passaged to culture flasks without MEFs; MA formed after 4 to 5 days in the absence of LIF or any additional MEF, and presented with multiple cell layer thickness over the few residual MEF cells (Figure 1). Typically the aggregates are round and have a well defined border. Afterwards, the MA layers are trypsinized and transferred to flasks or culture inserts where they lose their aggregate morphology and continue to proliferate (Figure 2).

The culture containing MA, and residual mitotically inhibited MEFs, were then trypsinized and seeded in 24-well plates fitted with culture inserts (Isopore PCF polyester Millicell[®] culture plate inserts, 2.3 cm² effective surface area, 10⁵ cells per insert) with or without extracellular matrix (ECM) component coating (Biocoat[®] inserts, Becton Dickinson Labware, Bedford, MA, USA). These components included: collagen type I (C-I), collagen type IV (C-IV), fibronectin (FN), and laminin (LN). In addition, cells were grown in the presence of one of the following human growth factors (Sigma Life Sciences, St. Louis, MO, USA): EGF, 400ng/ml; AR, 200ng/ml; transforming growth factor-β1 (TGFβ1), 0.4ng/ml; and, keratinocyte growth factor (KGF), 2ng/ml. The differentiated MA cultures were grown for another 10-16 days, during which time daily transmonolayer specific resistance (Ω•cm², see section 2.2.3) was measured using the Millicell-ERS[®] resistance system (Millipore Corp., Temecula, CA, USA) before and after incubation with test chemicals.

2.2.2. MTT cell viability assay—The acute cytotoxic effects of 19 chemicals (see Table 1) on cell viability were measured in confluent monolayers in 96-well plates, using the MTT assay (Dolbeare and Vanderlaan, 1994). This assay was originally described by Mosmann (1983), and modified as previously described (Schmidt *et al.* 2004;Konsoula and Barile, 2007). The

²Although ATCC does not specify passage level, we routinely grow and maintain cultures under 30. This allows for shorter intervals between passages to maintain undifferentiated status; observable changes in culture characteristics are also less likely to occur.

tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), is actively absorbed in a succinate-NADH⁺ mitochondrial-dependent reaction to yield a formazan product. The ability of the cells to reduce MTT provides an indication of mitochondrial activity that is interpreted as a metabolic marker for cell viability.

Cultures containing MA were grown in 96-well plates until confluent, in ES-DMEM (components are noted above, section 2.1) but in the absence of LIF, ECM, GFs, or any additional MEF. Confluent monolayers were exposed to increasing concentrations of each chemical (12 wells per concentration-group plus 1 control group; Table 1) for 24-h at 37°C in an atmosphere of 7.5% CO₂ in air. In the last hour of incubation, 10 µl MTT solution (5mg/ml in DMEM) was added to each well. The medium was replaced with 100 µl dimethylsulfoxide, agitated for 1 min at 25°C, and the absorbance was read at 550nm on the BioTek FL600[®] fluorescence/absorbance plate reader (BioTek Instruments Inc., Winooski, VT, USA). Cell viability is expressed as a percentage of the control group. Control groups consisted of cells in media (minus chemical) which were processed identically and incubated simultaneously as treated groups. Parallel sets of wells without cells were incubated as process blanks.

For all assays, dosage range-finding experiments were performed. Every experiment was repeated at least three times. For example, calculation of IC₅₀ value for each chemical is based on a minimum of 3 experiments performed at separate times, 8 groups (concentrations) per chemical (including control and blank), and 6 separate measurements (samples) per group, ($N = 3$; sample number of measurements = 144). When the IC₅₀ was not bracketed in the initial dosage range used for the chemical, the experiments were repeated and the concentrations were adjusted as necessary. Values in figures are expressed as percent of control groups. (Additional protocol details involving chemicals are noted below, Section 2.3).

2.2.3. Transepithelial electrical resistance (TEER) measurements—As noted above, cultures containing MA, and residual mitotically inhibited MEFs, were trypsinized and seeded in 24-well plates fitted with Biocoat[®] culture inserts with or without ECM component coating. For TEER experiments, control inserts were not coated and LIF was removed from all groups. In addition, the inserts were divided into several groups and the cultures were allowed to proliferate and differentiate in the presence of ES-DMEM plus one of the following human GFs per group—EGF (400ng/ml), AR (200ng/ml), TGFβ1 (0.4ng/ml), or KGF (2ng/ml)—for an additional 10 to 16 days. During this time daily transmonolayer³ specific resistance ($\Omega \cdot \text{cm}^2$) was measured. Results are detailed below and shown in Figure 3. Experiments were repeated and the N number generated as per the MTT assay (see Section 2.2.2 above).

2.2.4. Paracellular permeability (PP) studies—PP studies were performed as previously described (Konsoula and Barile, 2005 and 2007). Briefly, proliferating MA cultures were seeded onto 12-well transwell polycarbonate inserts as described above. Cultures were incubated with the chemicals, which were introduced in ES-DMEM on the apical side of the insert (in contact with the cell layer), for 24-h; low and higher molecular weight PP markers were introduced into the apical chamber in the last 90 min of incubation. The markers included [³H]-D-mannitol ([³H]-D; mw = 182, 17 Ci/mmol, Perkin-Elmer, Boston, MA, USA), lucifer yellow (LY, mw = 450, Sigma-Aldrich, St. Louis, MO, USA) and fluorescein-isothiocyanate dextran (FITC-dextran, mw = 40K-50K, Sigma-Aldrich). Also, in order to stabilize and account for changes in the osmolarity or pH of media from chemicals whose TEER IC₅₀ values exceeded 100mmol/L, the incubating media were adjusted with 100 µl, 1N NaOH and up to 25 µM HEPES buffer (applicable for salicylic acid, ibuprofen and dimethyl formamide (Table 1).

³The term *transmonolayer* is used here, as opposed to transepithelial, since the differentiation status of the MA is unknown. However, the TEER indication is widely accepted and understood.

For radioactive experiments, D-mannitol (0.1% w/v) was dissolved in ES-DMEM, supplemented with [^3H]-D (17 Ci/mmol), and added onto the apical side (0.5ml) of the insert to a final concentration of 1 mCi/L (Liu *et al.*, 1999). ES-DMEM without [^3H]-D was added to the basolateral side. At the end of the exposure period, an aliquot of basolateral medium was measured by liquid scintillation counting (Beckman LS5801 counter, Beckman Coulter Inc., Fullerton, CA, USA). Blanks (inserts without cells) and control groups (minus chemical) were monitored simultaneously. Background radioactivity was determined using DMEM, and dpm was calculated based on the instrument's counting efficiency (for [^3H] ~45%).

LY and FITC-dextran fluorescent indicators were used at concentrations of 1mg/ml in ES-DMEM and applied to the apical side of the insert, as per the radioactive experiments. At the end of the incubation and labeling period, an aliquot of the basolateral medium was collected and fluorescence intensity was measured with the BioTek FL600[®] fluorescence/absorbance microplate reader. Experimental and process (blank) controls were monitored simultaneously. The excitation and emission wavelengths for LY are 430nm and 540nm, and for FITC-dextran, 487nm and 518nm, respectively. Relative cell permeability was expressed as a percent of untreated control groups. Experiments were repeated and the *N* number generated as noted above (Section 2.2.2).

2.2.5. Gene detection for differentiation by RT-PCR—Total RNA was isolated from cell pellets using the Invitrogen Micro-to-Midi total RNA isolation kit[®] (Invitrogen Corp., Carlsbad, CA, USA). Gene primers include: Oct-4/POU5f1 (POU domain transcription factor for undifferentiated cells and trophectoderm layers), Afp (alpha fetoprotein for MA and EBs), Arnt (aryl hydrocarbon receptor nuclear translocator for differentiated cells), Egfr (epidermal growth factor receptor for differentiated cells), and Tgf β r2 (transforming growth factor, beta receptor II for transformed cells). Forward and reverse primer sequences for all transcripts were purchased from SuperArray Bioscience Corp. (Frederick, MD, USA) (Ginis *et al.*, 2004).

RT-PCR was performed with SuperScript[™]III one-step RT-PCR with platinum[®]Taq (Invitrogen Corp., Carlsbad, CA, USA), using 0.4 μg RNA in each reaction (Kebache *et al.*, 2002). PCR amplification was performed from a modification of the manufacturer's protocol to allow for gene quantification using 18S ribosomal internal standard (IS; Ambion Corp., Austin, TX, USA). PCR products were amplified using a 1-min hot start at 94°C, 1 cycle of 30 min at 50°C and 35 cycles of 15 s at 94°C, 30 s at 55°C, and 60 s at 72°C, and a final extension at 72°C for 10 min. Expression products were separated using 2% agarose gel containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide for 60 min at 100v. A 50 basepair DNA ladder (Invitrogen Corp.) was used to estimate the size of the amplified bands. Band intensity was measured using the FluoroChem 8000 Fluorescence Digital Imaging System[®] (FluoroChem Inc., Azusa, CA, USA). The bands were scanned for individual genes expressed in the PCR analysis and were compared to the IS applied to the gel simultaneously and under identical conditions as the treatment sample. The information was then used to calculate the ratios in the presence of combinations of ECM and GFs.

2.3. Additional chemical solubility and protocol details

The 19 test chemicals were purchased from Sigma-Aldrich; they were stored desiccated at either 4°C or -10°C according to supplier's instructions. Soluble chemicals were dissolved in ES-DMEM stock solutions and concentrations were derived from aliquots of the stock solutions. Solid chemicals that were insoluble in ES-DMEM, especially at the higher dosage levels, was improved by the addition of a solvent to the incubating medium as follows (with pH adjusted to 7.4): salicylic acid and ibuprofen—1N NaOH; propranolol, verapamil, doxorubicin, actinomycin—128 mmol/L (1% w/v) DMSO; quinine sulfate—100 mmol/L (0.5%

w/v) ethanol. In separate experiments we determined that none of the additives influenced control group cytotoxicity or paracellular permeability of [³H]-D-mannitol. In addition, ethanol (100 mmol/L) served as the negative control compound and is within ± 1 SE of the untreated control groups (100% of control cell viability). Matching concentrations of solvents were also added to the corresponding treatment groups where applicable.

2.4. Statistical Analysis

The 50% inhibitory concentrations (IC₅₀ values) were extrapolated from concentration-effect curves using linear regression analysis. The coefficient of determination (R^2), regression analysis, slopes and the t -statistic (one- or two-tailed paired Students' t -test with the more stringent equal variances assumption) were also calculated for each set of data.

In addition, calculated regressions for the mES cells were compared with the RC regression (ICCVAM, 2001) and with previous cytotoxicity data generated for Caco-2 and IEC-18 cells. The RC regression equation is based on the following formula:

$$\log(\text{LD}_{50}) = 0.435 \times \log(\text{IC}_{50}) + 0.625$$

where $R^2 = 0.67$ for 347 chemicals in the RC database (Halle *et al.*, 2000), and 0.97 for 11 of the 19 chemicals (ICCVAM, 2001) used in this study. If the regression line obtained with our model parallels the RC regression and is within $\pm \log 5$ interval, then the test is considered suitable to generate IC₅₀ data to use with the RC regression for estimating starting doses.

For all experiments, every figure data point or IC₅₀ value for each chemical was calculated from a minimum of 3 experiments performed at separate times, 8 groups per chemical (each representing individual concentrations, including control and blank), 6 separate measurements (sample wells) per group ($n = 3$; sample number of measurements = 144). Because of the high sample repetitions, the SE for each data point presented in the tables and figures are between 1 and 3%. In addition, propagation of mES cells on culture inserts was performed bi-monthly over the course of 2 years.

3. Results

3.1. Induction of formation of intact monolayers in culture inserts

Changes in TEER values for proliferating cultures of MA in culture inserts in the presence of GFs (inset: amphiregulin, EGF = epidermal growth factor, KGF = keratinocyte growth factor, LIF = leukemia inhibitory factor, TGF β 2 = transforming growth factor beta-receptor II) and 4 different ECM coatings (panels: A = collagen type I, B = collagen type IV, C = fibronectin, D = laminin) are illustrated in Figure 3. TEER values increased early in culture with FN and collagen-coated inserts, whereas in the absence of ECM or in the presence of LN, this process was delayed by several days. Also, highest TEER values, up to 700 Ω -cm², were achieved with FN and C-IV coated inserts. In all cases, LIF and MEFs prevented formation of intact monolayer structures, as evidenced by control baseline values.

TEER values of at least 500-700 Ω -cm² were achieved when MA were grown in the culture inserts for 10-16 days without MEF or LIF. The development of high resistance intact monolayers was accelerated and best demonstrated when inserts were coated with C-IV or FN and when the culture medium was supplemented with Kgf or Egf. The gradual increase and magnitude of the level of TEER is an important finding and suggests that proliferating MA, in the absence of MEF or LIF, differentiate to form intact confluent selectively permeable monolayers (baseline measurements for control cells were typically at 100-150 Ω -cm²).

3.2. Cytotoxicity Data

3.2.1. Comparison of PP data and TEER measurements—Figures 4, 5, and 6 illustrate results from PP experiments using PP transport markers. The concentrations used are based on the IC₅₀ data determined for the TEER experiments and range from .001 to 5-fold of IC₅₀ (Table 1). Each figure groups four representative chemicals with corresponding transport of permeability markers at concentrations based on the viability assays. The plots illustrate that paracellular transport of [³H]-D, as a PP marker, more closely parallels the fall in TEER than LY or FITC-dextran. In every instance, transport of [³H]-D to the basolateral surface is proportional to the decrease in transmonolayer specific resistance. PP data for the rest of the chemicals are not shown but mimic the graphs in Figures 4, 5 and 6.

3.2.2. Cytotoxicity testing with confluent mES cells: comparison of cell viability and PP data—Table 1 compares the results generated with the *in vitro* assays, and also contrasts them with standardized data from the RC database. The IC₅₀ values were generated from the MTT assay and TEER studies using confluent monolayers of mES cells. Cytotoxicity was determined using MTT cell viability assay in 96 well plates; TEER was measured in confluent monolayers grown on filter membranes, as described above. IC₅₀ values for each chemical were calculated from regression analyses of the plots of cell viability studies, the graphs of which are shown in Figure 7. This graph illustrates concentration-effect curves for 16 of the 19 chemicals using the 24-hr MTT assay. The test statistic (*t*), for the calculation of each line of best fit, were significant for all plots as were the coefficients of determination (*R*² values greater than 0.90 for all chemicals plotted, using log of concentration). Positive control chemical (actinomycin) represents the standard compound with the expected most toxic effect (Table 1). As noted above, ethanol did not show toxicity as the negative control chemical at 100 mmol/L (0.5% w/v). We have also previously reported the IC₅₀ for ethanol in a 24-hr MTT assay using human lung cells at greater than 500 mmol/L (Yang *et al.*, 2002).

Table 2 summarizes the statistical analysis of the data in Table 1. The *R*² and the slope (*m*) of the lines of best fit are indicated. *R*² measures the degree of correlation between the sets of data, while the slope is an indication of the deviation of the plot of experimental values from a 1:1 (mM:mM) relationship. ANOVA calculations revealed that significant differences exist among the data sets (*F*-significance <0.01); further statistical comparisons revealed significant differences between groups where indicated (* tables 2 and 3, one- or two-tailed paired Students' *t*-test, *P*<0.05,).

The data reveal that MTT, as a measure of cell viability, correlates well with TEER measurements (*R*² = 0.91). Cell viability, however, is a more sensitive indicator of cytotoxicity than TEER, as noted by the slope of the line (*m* = 0.08, significantly different at *P*<0.05, Table 2). This conclusion is based on the fact that while *R*² measures the degree of correlation between the sets of data, the slope is an indication of the deviation of the plot of experimental values from a 1:1 relationship. Thus, in the calculation of slope on a log scale, when *m* is less than 1.0, the line is shifted to the right and the *y*-values are lower than corresponding *x*-values. This suggests that mitochondrial activity is more likely to be altered before paracellular permeability is compromised. Our model, for the combination of AC and PP measurements, thus allows for the differentiation between the concentrations necessary for AC and those needed to interfere with the integrity of the intact monolayer structure.

3.2.3. Cytotoxicity testing with confluent mES cells: Comparison of cytotoxicity data with the *in vitro* IC₅₀ data and *in vivo* LD₅₀ RC database—Similarly, we compared the IC₅₀ values generated for 19 chemicals using the MTT assay and TEER measurements, with IC₅₀ values from the RC database (Table 1). The statistical analysis is tabulated in Table 2. Inspection of the data indicates that the MTT assay is more sensitive than

the RC IC₅₀ and LD₅₀ values, based on the slope of the lines ($m=0.07$ and 0.35 , respectively). Although these comparisons are not significantly different, the slopes of the MTT values are consistently shifted to the right of the RC IC₅₀ and LD₅₀ values, indicating lower numbers (figures for Table 2 are not included but are similar to graphs of Figure 8). The values also do not correlate well ($R^2=0.28$ and 0.38 , respectively). In addition, TEER values were equivalent to the IC₅₀ data from the RC database ($m=1.04$), and less sensitive than the reported LD₅₀s ($m=5.08$). It is not surprising, therefore, that TEER measurements were significantly different from RC LD₅₀s (Table 2; $* = P<0.05$; it should be noted that the IC₅₀ values from the RC database use the neutral red uptake assay as a cell viability marker and the 3T3 cell line).

3.3. Cytotoxicity testing with confluent mES cells: Comparison of cytotoxicity data with Caco-2 and IEC-18 in vitro IC₅₀ data

Table 3 compares the IC₅₀ MTT and TEER data generated with the mES cells (from Table 1) with previously published data obtained from Caco-2 and IEC-18 cells using identical methodologies (Konsoula and Barile, 2005 and 2007, respectively). The statistical analyses are elucidated when the corresponding graphs are plotted (Figure 8). High correlations (R^2) are obtained when the same methodologies are compared between mES cells and the other cell lines. In particular, mES cells are significantly different (*) and more sensitive to the cell viability effects of the chemicals ($m=0.36$) than Caco-2 cells. Moreover, mES cells are more responsive to effects on cell viability when compared to IEC-18 cells ($m=0.26$), although the paired comparisons of the IC₅₀ data are not statistically different. TEER IC₅₀ measurements are almost identical between the cell lines (Figures 8c and d, and Table 3), suggesting that toxic insult to intact monolayers of MA cultures are on the order and magnitude of continuous human intestinal cell lines.

3.4. Gene expression as evidence of differentiation status of MAs using RT-PCR

In order to gauge the state of differentiation of MA in culture inserts, we used several gene transcripts that distinguish undifferentiated cells from their differential progeny. This also enabled us to correlate differentiation of MA cultures in inserts with the ability of the monolayers to produce high TEER resistance membranes.

Differential expression of gene primers was quantified (Table 4) and compared to an 18S ribosomal internal standard (Figures 9 and 10). Figure 9 shows representative expression of gene transcripts from MA cultures grown on inserts for 14-16 days that were coated with collagen type-IV in the presence of Kgf. Egfr expression is highest among the genes amplified, compared to IS (lane 3; ratio of Egfr/IS = 2.2, Table 4), while Tgfβr2 expression (lane 5, ratio=0.8) and Afp (lane 1, ratio=0.8) were lower (Egfr and Tgfβr2 are expressed in differentiated cells and transformed cells, respectively, whereas Afp is expressed in transitional systems such as MA). Lowest expression was demonstrated for Oct-4, a marker for undifferentiated mES cells (lane 4, ratio=0.5). Consistent with these results and as a biological control, MEFs showed the highest expression of Tgfβr2 (5.2, Table 4), as a marker for immortal, transformed cells.

Figure 10 illustrates Kgf-induced expression from MA cultured on FN (lanes 1-5) and LN (lanes 7-11; lane 6 = 50 bp DNA ladder). Expression for Egfr was higher in the presence of FN (lane 3, ratio=2.5) and LN (lane 9, ratio=2.8) with lower expression of Oct-4 and Tgfβr2 (lanes 4 and 5, ratio=1.1 and 1.0, respectively). EGF, however, in the presence of laminin, induces a heterogeneous pattern of gene expression since all of the ratio values are higher. This suggests that these cultures have not uniformly transformed from the MA stage. For instance, Egfr (lane 9, ratio=5.7) and Oct-4 expression (lane 10, 3.0), markers for differentiated and undifferentiated cells respectively, demonstrated high band intensities with LN-coated inserts

(Figure 10 and Table 4). In FN coated inserts, calculation for Afp/IS ratio (an MA marker, lane 1) is high (2.8).

4. Discussion

When ES cells are allowed to differentiate in suspension culture, they form spherical multicellular aggregates (EBs) that contain a variety of cell types (Yamada *et al.*, 2002). Similarly, in the absence of LIF or MEF after 4 to 5 days of growth, mES cells form rounded well defined aggregates resembling EBs, containing multiple cell layers over the few remaining MEF cells (Figure 1). As the MA colonies propagate in the absence of feeder layers and LIF, they lose their aggregate morphology and migrate peripherally (Figure 2).

Limited evidence exists for the differentiation of mES cells into specific populations, such as hematopoietic cells (Potocnik *et al.*, 1997), cardiomyocytes (Klug *et al.*, 1996), smooth muscle cells (Drab *et al.*, 1997), and neurons (McDonald *et al.*, 1999). Keller *et al.* (2004) describe the induction of epithelial- or epidermal-specific gene expression and differentiation using a combination of GFs plus basal lamina substrata in human ES cells. Based on these reports, we calculated that if mES cells are allowed to proliferate on porous inserts coated with ECM substrata in the absence of MEF layers or LIF, we could direct the cells to differentiate and form confluent monolayers with high TEER resistance. In our first attempts toward this end, we omitted MEF, LIF, and GFs with the intention of inducing spontaneous differentiation and formation of TJs based on their attachment to a basement membrane-coated culture insert. Generation of monolayers with high TEER values was not extensive under these conditions (maximum TEER values reached 450 Ω -cm², Figure 3, minus LIF), indicating minimal formation of TJs. Thus, in the absence of GFs, the lack of production of higher resistance monolayers prompted us to incorporate GFs into the protocol. These conditions generated intact monolayers with TEER values measured at 650-700 Ω -cm², suggesting intact confluent monolayers with formation of TJs.

Results from RT-PCR experiments are in agreement with the TEER studies and indicate that MA cultures differentiate into specific cell lineages (epidermal or epithelial cell lines) in 30 to 40% less time than expected in the absence of GFs and ECM components. Highest expression of *Egfr* transcripts, as a marker of epidermal differentiation, also confirms the high TEER values obtained when culture inserts are supplemented with EGF or KGF (Figure 3).

Further evidence for the influence of ECM and GFs on differentiation of MA in culture inserts is summarized as RT-PCR data in Table 4. The data reveal that FN and LN, in the presence of KGF or EGF, induce higher expression of genes (*Egfr* and *Arnt*) corresponding to the differentiation of MA cultures with the highest measured TEER values. In addition, Oct-4 expression (Table 4) is down-regulated for the corresponding combination of GF/ECM, suggesting that by days 14-16, the MA cultures have differentiated, while *Tgf β 2* expression is consistent with the presence of transformed cells ("MEF cells only" as biological controls). Thus semi-quantitative RT-PCR results suggest that differentiation of MA cultures in the presence of extracellular factors is consistent with the gradual increase in TEER values during the culture period. Although there appears to be progressive differentiation of MA toward an epidermal cell lineage, expression of Oct-4 and Afp in the same system suggest that the cultures are heterogeneous and correspond to various stages of differentiation. It is interesting to note that while the cultures appear to express markers of epidermal lineage, differentiation to an epithelial line has not been excluded.

The mechanism by which GFs and ECM influence cell differentiation is the subject of much interest. GFs are capable of inducing linear specific differentiation. For instance, EGF is a mitogenic polypeptide that allows or induces differentiation into ectoderm (including skin) and

mesoderm (Gritti *et al.*, 1999), while KGF is a cell-specific mitogen responsible for normal proliferation and differentiation of epithelial cells (Visco *et al.*, 2004). The TGF β family of proteins, including the activins and the inhibins, bind to different cell surface receptors with varying affinities. They induce proliferation of mesenchymal and epithelial cell types while demonstrating anti-proliferative effects on endothelia, macrophages and lymphocytes (Mercado-Pimentel and Runyan, 2007). Variants of the Tgf β r genes result in decreased TGF β -mediated growth inhibition and an overall increase in cancer risk (Kalamani and Pasche, 2004). Amphiregulin (AR) is a member of the EGF family. AR is synthesized as a precursor; it is shed from the plasma membrane by metalloproteases and is produced in a variety of tumors. Thus, AR is a pro-regenerative and survival growth factor that displays a non-redundant role in cancer development, particularly during acute liver injury (Berasain *et al.*, 2007). In our experiments, therefore, it was reasonable to infer that enrichment of MA in culture with several specific GFs would provide an environment for cellular expansion, improved survival, or induced differentiation. Thus, our results suggest that GFs, particularly KGF in the presence of C-IV substratum, induce differentiation of MA to form confluent monolayers, with the subsequent formation of TJs. This accounts for the high TEER values.

We obtained high regression values between MTT IC₅₀ values and TEER measurements (Table 2), indicating that PP varies indirectly with cell viability—i.e. as cytotoxicity increases, TEER decreases proportionately. According to the regression standards for the RC database, cytotoxicity is predicted by our system. Although regression values are low, the slopes for all MTT assays vs. the RC IC₅₀ values, RC LD₅₀ values, and TEER measurements, are less than 1.0, suggesting that cell viability measurements are more sensitive than the latter. Consequently, computation of R^2 alone is not sufficient for determining sensitivity of *in vitro* systems.

The results obtained from MA grown on cell culture inserts reveal that they form intact monolayers with electrical resistance comparable to that of Caco-2 and greater than that of IEC-18 cells. Differentiated cultures also demonstrate that there is a direct correlation between the disruption of the structural barrier and PP. Increased transmonolayer passage of markers results when membrane integrity is compromised. Also, as with previous cell lines, mES cells are as sensitive to toxic insult as immortal colon and finite small intestine cells. Moreover, all three cell lines reveal similar TEER responses to chemical insult. Interestingly, analysis of these and previously published data demonstrates that mES and IEC-18 cells are permeable to all PP markers in contrast to Caco-2, the latter of which displays favorable passage of [³H]-D only (Konsoula and Barile, 2007). This may be explained by variable expression of transporter/efflux systems present in Caco-2 cells which have been shown to alter paracellular and transcellular passage of compounds, especially higher molecular weight chemicals such as FITC-dextran (Behrens and Kissel, 2003).

Paracellular transport of TJ markers, especially low-molecular weight [³H]-D, correlates precisely with decreases in TEER. Since IC₅₀ calculations for MTT experiments were generally lower than TEER measurements, and PP of TJ markers increases systematically with a fall in TEER, it is reasonable to conclude that cellular viability is compromised before PP is affected. Thus, the mES/MA culture insert model confirms our previous observations using immortal human colon cells and normal rat intestinal epithelial cells—i.e., the system allows for the distinction between the concentrations necessary for AC and those needed to interfere with PP. It is also important to note that knowledge of a compound's intestinal permeability or absorption is not necessarily useful to classify cytotoxicity [we have previously shown that comparison of 24-hr MTT IC₅₀ data for Caco-2 cells with available permeability indexes for the 19 compounds tested reveals no correlation, suggesting that permeability indexes and cytotoxicity are not necessarily related (Ingels *et al.*, 2004; Konsoula and Barile, 2005; Ruell *et al.*, 2003)].

Our findings for transformation of mES cells to MA, and subsequent induction of differentiation to form intact monolayers with TJ formation, provides new possibilities for obtaining developmentally regulated monolayer structures for the replacement of epithelia lost to pathological complications. In addition, orientation of undifferentiated stem cells toward lineages with distinct programmed functions, presents the potential for understanding epithelial polarity, compartmentation, and barrier function. Most importantly, manipulation of ES cells toward specific lineages presents a unique investigative opportunity for measuring the *in vitro* response to chemicals for toxicokinetic and cytotoxicity modeling. In particular, the construction of intact monolayers with TJ formation may be of predictive value for epigenetic studies related to *in vivo* gastrointestinal development. It is in this specialized manipulation associated with stem cell differentiation wherein the advantage lies beyond other cell culture models, particularly since the model more closely resembles the *in vivo* situation than Caco-2 or IEC-18 cells. Gene expression and immunohistochemical experiments are currently aimed at categorizing the stage of differentiation of cells in the presence of GFs and ECM, and their influence on TJ formation.

Acknowledgements

This work was supported in part by grants from the International Foundation for Ethical Research (IFER, Chicago, IL, USA) and the Alternatives Research & Development Foundation (ARDF, Jenkintown, PA, USA). Parts of this study were presented at the annual meetings of the Society for *In Vitro* Biology, June 2006, and the U.S. Society of Toxicology, March 2007.

References

- Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI. Embryonic stem cells express neuronal properties *in vitro*. *Developmental Biology* 1995;168:342–357. [PubMed: 7729574]
- Barile, FA. Cell culture methods for acute toxicity testing. In: Barile, FA., editor. *Principles of Toxicology Testing*. Taylor & Francis LLC; Boca Raton, FL, USA: 2007. p. 151-174.
- Behrens I, Kissel T. Do cell culture conditions influence the carrier-mediated transport of peptides in Caco-2 monolayers? *European Journal of Pharmaceutical Sciences* 2003;19:433–442. [PubMed: 12907294]
- Berasain C, Castillo J, Perugorría MJ, Prieto J, Avila MA. Amphiregulin: A new growth factor in hepatocarcinogenesis. *Cancer Letters* 2007;254:30–41. [PubMed: 17321672]
- Bradley A, Evans M, Kaufman M, Robertson E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* 1984;309:255–256. [PubMed: 6717601]
- Carriere V, Chambaz J, Rousset M. Intestinal responses to xenobiotics. *Toxicology in Vitro* 2001;15:373–378. [PubMed: 11566566]
- Curren R, Bruner L, Goldberg A, Walum E. 13th meeting of the Scientific Group on Methodologies for the safety evaluation of chemicals (SGOMSEC): Validation and acute toxicity testing. *Environmental Health Perspectives* 1998;106:419–425. [PubMed: 9599688]
- Dolbeare, F.; Vanderlaan, M. Techniques for measuring cell proliferation. In: Tyson, CA.; Frazier, JM., editors. *In Vitro Toxicity Indicators, Methods in Enzymology*. 1B. Academic Press; San Diego: 1994. p. 178-200.
- Drab M, Haller H, Bychkov R, Erdmann B, Lindschau C, et al. From totipotent embryonic stem cells to spontaneously contracting smooth muscle cells: a retinoic acid and db-cAMP *in vitro* differentiation model. *FASEB Journal* 1997;11:905–915. [PubMed: 9285489]
- Evans M, Kaufman M. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981;292:154–156. [PubMed: 7242681]
- Furue M, Okamoto T, Hayashi Y, Okochi H, Fujimoto M, et al. Leukemia inhibitory factor as an anti-apoptotic mitogen for pluripotent mouse embryonic stem cells in a serum-free medium without feeder cells. *In Vitro Cell and Developmental Biology–Animal* 2005;41:19–28.
- Genschow E, Scholz G, Brown N, Piersma A, Brady M, et al. Development of prediction models for three *in vitro* embryotoxicity tests in an ECVAM validation study. *In Vitro and Molecular Toxicology* 2000;13:51–66. [PubMed: 10900407]

- Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, et al. Differences between human and mouse embryonic stem cells. *Developmental Biology* 2004;269:360–380. [PubMed: 15110706]
- Gritti A, Frolichsthal-Schoeller P, Galli R, Parati EA, Cova L, et al. Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain. *Journal of Neuroscience* 1999;19:3287–3297. [PubMed: 10212288]
- Halle W. Toxicity testing in cell cultures to predict acute toxicity (LD₅₀) and to reduce testing in animals. *Alternatives to Laboratory Animals* 2003;31:89–198. [PubMed: 15612878]
- Halle W, Spielmann H, Liebsch M. Prediction of human lethal concentrations by cytotoxicity data from 50 MEIC chemicals. *ALTEX* 2000;17:75–79. [PubMed: 11085862]
- He YL, Murby S, Warhurst G, Gifford L, Walker D, et al. Species differences in size discrimination in the paracellular pathway reflected by oral bioavailability of polyethylene glycol and D-peptides. *Journal of Pharmaceutical Sciences* 1998;87:626–633. [PubMed: 9572915]
- ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). Report of the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity. National Institute of Environmental Health Sciences; Research Triangle Park, NC, USA: 2001 [December 2, 2007]. NIH Publication 01-4499<http://ntp-apps.niehs.nih.gov/iccvampb/searchDoc.cfm>
- ICCVAM. Guidance document on using *in vitro* data to estimate *in vivo* starting doses for acute toxicity. National Institute of Environmental Health Sciences; Research Triangle Park, NC: 2001 [December 2, 2007]. NIH Publication 01-4500<http://ntp-apps.niehs.nih.gov/iccvampb/searchDoc.cfm>
- Ingels F, Beck B, Oth M, Augustijns P. Effect of simulated intestinal fluid on drug permeability estimation across Caco-2 monolayers. *International Journal of Pharmaceutics* 2004;274:221–232. [PubMed: 15072798]
- Ishikawa T, Nakayama S, Nakagawa T, Horiguchi K, Misawa H, et al. Characterization of *in vitro* gutlike organ formed from mouse embryonic stem cells. *American Journal of Physiology–Cellular Physiology* 2004;286:C1344–1352.
- Kaklamani VG, Pasche B. Role of TGF- β in cancer and the potential for therapy and prevention. *Expert Review of Anticancer Therapy* 2004;4:649–661. [PubMed: 15270668]
- Kebache S, Zuo D, Chevet E, Larose L. Modulation of protein translation by Nck-1. *Proceedings of the National Academy of Sciences USA* 2002;99:5406–5411.
- Keller MS, Ezaki T, Guo R-J, Lynch JP. Cdx1 or Cdx2 expression activates E-cadherin-mediated cell-cell adhesion and compaction in human COLO 205 cells. *American Journal of Physiology–Gastrointestinal and Liver Physiology* 2004;287:G104–G114. [PubMed: 14977637]
- Kirkland SC, Henderson K. Collagen IV synthesis is restricted to the enteroendocrine pathway during multilineage differentiation of human colorectal epithelial stem cells. *Journal of Cell Science* 2001;114:2055–2064. [PubMed: 11493641]
- Klug MG, Soonpaa MH, Koh GY, Field LJ. Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. *Journal of Clinical Investigation* 1996;98:216–224. [PubMed: 8690796]
- Konsoula R, Barile FA. Correlation of *in vitro* cytotoxicity with paracellular permeability in Caco-2 cells. *Toxicology In Vitro* 2005;19:675–684. [PubMed: 15896555]
- Konsoula R, Barile FA. Correlation of *in vitro* cytotoxicity with paracellular permeability in mortal rat intestinal cells. *Journal of Pharmacological and Toxicological Methods* 2007;55:176–183. [PubMed: 16891127]
- Kuwahara M, Ogaeri T, Matsuura R, Kogo H, Fujimoto T, Torihashi S. *In vitro* organogenesis of gut-like structures from mouse embryonic stem cells. *Neurogastroenterology and Motility* 2004;16:14–18. [PubMed: 15065998]
- Liu DZ, LeCluyse EL, Thakker DR. Dodecylphosphocholine-mediated enhancement of paracellular permeability and cytotoxicity in Caco-2 cell monolayers. *Journal of Pharmaceutical Sciences* 1999;88:1161–1168. [PubMed: 10564065]
- McDonald JW, Liu XZ, Qu Y. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nature Medicine* 1999;12:1410–1412.

- Mercado-Pimentel ME, Runyan RB. Multiple transforming growth factor-beta isoforms and receptors function during epithelial-mesenchymal cell transformation in the embryonic heart. *Cells Tissues Organs* 2007;185:146–156. [PubMed: 17587820]
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* 1983;65:55–63. [PubMed: 6606682]
- Potocnik AJ, Kohler H, Eichmann K. Hemato-lymphoid *in vivo* reconstitution potential of subpopulations derived from *in vitro* differentiated embryonic stem cells. *Proceedings of the National Academy of Sciences USA* 1997;94:10295–10300.
- Quaroni A, Hochman J. Development of intestinal cell culture models for drug transport and metabolism studies. *Adv Drugs Delivery Rev* 1996;22:3.
- Rohwedel J, Maltsev V, Bober E, Arnold HH, Hescheler J, Wobus AM. Muscle cell differentiation of embryonic stem cells reflects myogenesis *in vivo*: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. *Developmental Biology* 1994;164:87–101. [PubMed: 8026639]
- Ruell JA, Tsinman KL, Avdeef A. PAMPA—a drug absorption *in vitro* model-5. Unstirred water layer in iso-pH mapping assays and pK_a^{flux} -optimized design (*pOD*-PAMPA). *European Journal of Pharmaceutical Sciences* 2003;20:393–402. [PubMed: 14659483]
- Schmidt CM, Cheng CN, Marino A, Konsoula R, Barile FA. Hormesis effect of trace metals on cultured normal and immortal human mammary cells. *Toxicology and Industrial Health* 2004;20:57–68. [PubMed: 15807409]
- Spielmann H, Genschow E, Leibsch M, Halle W. Determination of the starting dose for acute oral toxicity (ID_{50}) testing in the up-and-down procedure (UDP) from cytotoxicity data. *Alternatives to Laboratory Animals* 1999;27:957–966.
- Visco V, Belleudi F, Marchese C, Leone L, Aimati L, et al. Differential response to keratinocyte growth factor receptor and epidermal growth factor receptor ligands of proliferating and differentiating intestinal epithelial cells. *Journal of Cellular Physiology* 2004;200:31–44. [PubMed: 15137055]
- Wiles MV, Keller G. Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development* 1991;111:259–67. [PubMed: 1893864]
- Yamada T, Yoshikawa M, Takaki M, Torihashi S, Kato Y, et al. *In vitro* functional gut-like organ formation from mouse embryonic stem cells. *Stem Cells* 2002;20:41–49. [PubMed: 11796921]
- Yang A, Cardona DL, Barile FA. Subacute cytotoxicity testing with cultured human lung cells. *Toxicology In Vitro* 2002;16:33–39. [PubMed: 11812637]

Abbreviations

³H-D	[³ H]-D-mannitol
AC	acute cytotoxicity
Afp gene (gene primers in lower case)	alpha fetoprotein
AR	amphiregulin
Arnt gene	aryl hydrocarbon receptor nuclear translocator
C-I	collagen type I
C-IV	collagen type IV

ECM	extracellular matrix
EGF	epidermal growth factor
Egfr gene	epidermal growth factor receptor
FITC	fluorescein isothiocyanate
FN	fibronectin
LN	laminin
GFs	growth factors (upper case)
IC₅₀	inhibitory concentration 50%
IS	internal standard
KGF	keratinocyte growth factor
LIF	leukemia inhibitory factor
LY	lucifer yellow
MA	multicellular aggregates
MEF	mouse embryonic fibroblasts
mES	mouse embryonic stem (cells)
Oct-4 gene	Oct-4/POU domain transcription factor
PP	paracellular permeability
RC	Registry of Cytotoxicity
TEER	transepithelial electrical resistance or transmonolayer specific electrical resistance
Tgfβr2 gene	

transforming growth factor beta-receptor II

TJ

tight junction

Fig. 1. mES cells differentiate into rounded multicellular aggregates (MA) after removal of LIF and in the presence of only “residual” MEF. The aggregates resemble EBs typically seen in suspension culture, are round and have a well defined border.

Fig. 2. Further culture in the absence of feeder layers and LIF induce MA to lose their aggregate morphology, as cells migrate to the periphery with continued proliferation.

Fig. 3.

Changes in TEER values for proliferating cultures of MAs in culture inserts in the presence of GFs (see insets) and 4 different ECM coatings (panels A, B, C, D). TEER values increased early in culture with FN and collagen-coated inserts, whereas in the absence of ECM or in the presence of LN, this process is delayed by several days. Experiments were repeated at least 3 times ($n \geq 3$).

Fig. 4.

Effect of (a) acrylamide, (b) trichloroforon, (c) sodium dichromate dihydrate and (d) verapamil, on PP in confluent monolayers of MAs. Concentrations are determined empirically and based on IC₅₀ measurements generated from MTT and TEER experiments (range 0.001 to 5-fold, Table 1). Scale for % of control for PP markers (bars) are on left axis; for TEER measurements (solid line), scale is on right axis. All control values are set at 100%. FITC = fluorescein isothiocyanate-dextran, LY = lucifer yellow, ^3H = [^3H]-D-mannitol, TEER = transmonolayer specific electrical resistance.

Fig. 5.

Effect of (a) actinomycin, (b) cadmium chloride, (c) cupric sulfate, and (d) doxorubicin, on PP in confluent monolayers of MAs. Concentrations are determined empirically and based on IC₅₀ measurements generated from MTT and TEER experiments (range .001 to 5-fold, Table 1). Scale for % of control for PP markers (bars) are on left axis; for TEER measurements (solid line), scale is on right axis. All control values are set at 100%. FITC = fluorescein isothiocyanate-dextran, LY = lucifer yellow, ³H = [³H]-D-mannitol, TEER = transmonolayer specific electrical resistance.

Fig. 6.

Effect of (a) dimethylformamide, (b) glycerol, (c) manganese chloride, and (d) lithium sulfate, on PP in confluent monolayers of MAs. Concentrations are determined empirically and based on IC₅₀ measurements generated from MTT and TEER experiments (range .001 to 5-fold, Table 1). Scale for % of control for PP markers (bars) are on left axis; for TEER measurements (solid line), scale is on right axis. All control values are set at 100%. FITC = fluorescein isothiocyanate-dextran, LY = lucifer yellow, ³H = [³H]-D-mannitol, TEER = transmonolayer specific electrical resistance.

Fig. 7.

Concentration-effect curves for 16 of the 19 chemicals using the 24-hr MTT assay. The test statistic (t) was calculated for each line of best fit, and was determined to be significant for all plots. Coefficients of determination (R^2 values) were greater than 0.90 for all chemicals. Positive control chemical (actinomycin) represents the standard compound with the most toxic effect (Table 1); ethanol did not show toxicity as the negative control chemical at 100 mmol/L (plot not shown since it is off scale).

Fig. 8.

MTT and TEER IC₅₀ values in confluent differentiated mES cells vs. corresponding IC₅₀ in Caco-2 and IEC-18 cells previously published (Konsoula & Barile, 2005, 2007, respectively). All experiments are 24-h exposures and were performed under identical conditions. The dotted lines represent theoretical 1:1 correlations. All axis values are in mmol/L.

Fig. 9.

Expression of transcripts (lanes 1-5) for MA cultures grown on inserts coated with collagen IV (C-IV) in the presence of KGF; (lanes: 1. Afp=alpha fetoprotein; 2. Arnt=aryl hydrocarbon receptor nuclear translocator; 3. Egfr=epidermal growth factor receptor; 4. Oct-4=POU domain, class 5, transcription factor 1; 5. Tgfβr2=transforming growth factor, beta receptor II; 6. 50 bp DNA ladder). Ratio of Egfr expression to internal standard (IS) was highest among the genes amplified (2.2), followed by Tgfβr2 (0.8), Afp (0.8), Arnt (0.5), and OCT-4 (0.5) (see also Table 4, C-IV, KGF).

Fig. 10.

Expression of transcripts for MA cultures grown on inserts coated with fibronectin (FN; lanes 1-5: 1. Afp, 2. Arnt, 3. Egfr, 4. Oct-4, 5. Tgf β 2) or laminin (lanes 7-11: 7. Afp, 8. Arnt, 9. Egfr, 10. Oct-4, 11. Tgf β 2; lane 6 = 50 bp DNA ladder), in the presence of KGF. Ratio of Egfr expression to internal standard (IS) was highest among the genes amplified, in the presence of FN (lane 3; Egfr/IS=2.5) and laminin (lane 9; Egfr/IS=2.8), with lower expression of Oct-4 in the presence of FN (lane 4; OCT-4/IS=1.1) and laminin (lane 10, OCT-4/IS=1.9) (see also Table 4, FN, LN, KGF).

Table 1

Comparison of IC₅₀ data (mmol/L) for mES cells using the MTT assay and TEER measurements, with IC₅₀ calculations (mmol/L) and LD₅₀ values (mmol/Kg) from the RC database for the same chemicals

Chemicals	MTT 24-h	TEER 24-h	RC IC ₅₀	RC LD ₅₀
acrylamide	5.03	21	1.61	2.39
actinomycin	0.0009	0.08	0.00008	0.0057
antipyrine	13	130	11.6	9.56
cadmium chloride	0.1	0.14	0.0064	0.48
cupric sulfate	0.4	0.5	0.33	1.2
dimethyl formamide	69.9	700	114	38.3
doxorubicin	0.008	0.05	0.00033	1.2
glycerol	34.68	588	624.0	137
Ibuprofen	0.145	0.1	0.52	4.89
lithium sulfate	9	97	34	10.8
manganese chloride	1.1	7	0.13	7.5
niacinamide	25.1	158	44	28.7
nickel chloride	0.277	1.0	0.27	0.81
propranolol	0.056	0.08	0.12	1.59
quinine HCl	0.084	0.11	0.075	1.72
salicylic acid	0.95	0.74	3.380	6.45
sodium dichromate *	0.075	0.048	0.00093	0.19
trichloroforon	0.96	2.08	0.27	1.75
verapamil HCl	0.09	0.44	0.10	0.22

^a From the Registry of Cytotoxicity (RC) database (ICCVAM, 2001);

* dihydrate salt.

Table 2

Statistical comparison of MTT and TEER IC₅₀ data from Table 1, and with IC₅₀ data from the RC database for the 19 reference chemicals^a

Data Comparisons (Y vs. X)	R^2	m
24h MTT (mES) vs. 24h TEER (mES) *	0.91	0.08
24h MTT (mES) vs. RC IC ₅₀	0.28	0.07
24h MTT (mES) vs. RC LD ₅₀	0.38	0.35
24h TEER (mES) vs. RC IC ₅₀	0.54	1.04
24h TEER (mES) vs. RC LD ₅₀ *	0.63	5.08

^aCalculations based on values obtained from the RC database (ICCVAM, 2001). R^2 = coefficient of determination for the regression analysis; m = slope of line of best fit.

* $P < 0.05$ (one- or two-tailed paired Students' t -test with the more stringent equal variances assumption).

Table 3

Statistical comparison of MTT and TEER IC₅₀ data from Table 1, and with IC₅₀ data from Caco-2 and IEC-18 cells for the 19 reference chemicals^a

Data Comparisons (Y vs. X)	R^2	M
24h MTT (mES) vs. 24h MTT (Caco-2) [*]	0.93	0.36
24h TEER (mES) vs. 24h TEER (Caco-2)	0.93	0.87
24h MTT (mES) vs. 24h MTT (IEC-18)	0.72	0.26
24h TEER (mES) vs. 24h TEER (IEC-18)	0.92	0.75

^a Comparisons based on cytotoxicity data for Caco-2 and IEC-18 cells previously published (Konsoula & Barile, 2005 and 2007, respectively). R^2 = coefficient of determination for the regression analysis; m = slope of line of best fit.

* $P < 0.05$ (one- or two-tailed paired Students' t -test with the more stringent equal variances assumption).

Ratios of gene expression to internal standard (IS) using RT-PCR of cultured MA in the presence of extracellular matrix (ECM) and/or growth factor (GF) after 14-16 days on culture inserts.

Table 4

ECM	GF	Afp/IS	Arnt/IS	Egfr/IS	Oct-4/IS	Tgfb α 2/IS
FN	KGf	1.3	1.5	2.5	1.1	1.0
	EGF	2.8	1.3	1.8	0.9	1.8
LN	KGf	1.2	1.5	2.8	2.9	1.3
	EGF	1.8	2.5	5.7	3.0	2.5
C-I	KGf	0.8	1.2	2.3	0.9	0.9
	EGF	0.6	0.7	1.0	0.7	0.9
C-IV	KGf	0.8	0.5	2.2	0.5	0.8
	EGF	0.9	0.8	1.1	0.7	0.8
MEF cells only	-	2.0	3.0	3.9	0.7	5.2

Values represent the average of 2 or 3 experiments. Gene primers: Afp = alpha fetoprotein, Arnt = aryl hydrocarbon receptor nuclear translocator, Egfr = epidermal growth factor receptor, Oct-4 = POU domain, class 5, transcription factor 1, Tgfb α 2 = transforming growth factor, beta receptor II; GF: EGF = epidermal growth factor, KGf = keratinocyte growth factor; ECM: FN = fibronectin, LN = laminin, C-I/IV = collagen types I/IV, MEF = mouse embryonic fibroblast feeder layers.