Messenger RNA Expression of Transporter and Ion Channel Genes in Undifferentiated and Differentiated Caco-2 Cells Compared to Human Intestines

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Purpose. The purpose of this work was to study the influence of cell differentiation on the mRNA expression of transporters and channels in Caco-2 cells and to assess Caco-2 cells as a model for carrier-mediated drug transport in the intestines.

Method. Gene mRNA expression was measured using a custom-designed microarray chip with 750 deoxyoligonucleotide probes (70mers). Each oligomer was printed four times on poly-lysine-coated glass slides. Expression profiles were expressed as ratio values between fluorescence intensities of Cy3 and Cy5 dye-labeled cDNA derived from poly(A) + RNA samples of Caco-2 cells and total RNA of human intestines.

Results. Significant differences in the mRNA expression profile of transporters and channels were observed upon differentiation of Caco-2 cells from 5 days to 2 weeks in culture, including changes for MAT8, S-protein, and Nramp2. Comparing Caco-2 cells of different passage number revealed few changes in mRNAs except for GLUT3, which was down-regulated 2.4-fold within 13 passage numbers. Caco-2 cells had a similar expression profile when either cultured in flasks or on filters but differed more strongly from human small and large intestine, regardless of the differentiation state of Caco-2 cells. Expression of several genes highly transcribed in small or large intestines differed fourfold or more in Caco-2 cells.

Conclusions. Although Caco-2 cells have proven a suitable model for studying carrier-mediated transport in human intestines, the expression of specific transporter and ion channel genes may differ substantially.

KEY WORDS: transporters; ion channels; microarrays; Caco-2 cells; intestinal drug transport.

INTRODUCTION

Drug absorption appears to occur predominantly via passive transcellular and paracellular transport mechanisms (1). However, recent studies indicate that carrier-mediated drug transport may play a more important role than previously thought. Sequencing of the human genome has suggested the presence of 406 genes encoding ion channels and 883 encoding transporters, of which 350 are intracellular transporters (2). Generally, these proteins establish the electrochemical gradient across membranes and provide the means for transporting amino acids, dipeptides, monosaccharides, monocarboxylic acids, organic cations, phosphates, nucleosides, and water-soluble vitamins (3,4). Frequently, transporters play a direct role in drug absorption, in particular from the intestines. The hPept1 transporter for instance is responsible for the carrier-mediated uptake of various peptide-like drugs, such as cephalexin, Angiotonsin converting enzyme (ACE) (1) inhibitors, and 5′-amino acid esters of the antiviral nucleosides acyclovir and AZT (5,6). L-Val-acyclovir is a prodrug for which the bioavailability was remarkably increased by a drug carrier, i.e., hPepT1. Further examples include pravastatin and salicylic acid, which are transported by a monocarboxylic acid transporter (7,8). However, transporters can also limit drug absorption through secretion into the intestinal lumen. P-glycoprotein, a member of the ABC superfamily (ABCB1, MDR1) is a prominent efflux pump, which limits the uptake of substrates, such as digoxin and cyclosporin (9,10). Further members of the ABC family might also contribute to reduced drug bioavailability.

Human adenocarcinoma cells, Caco-2, derived from a colon carcinoma, serve as a common *in vitro* model for estimating the fraction of compound absorbed via the intestinal tracts (11). The majority of drugs were thought to be passively transported. The fraction of a dose absorbed *in vivo* correlates significantly with the permeability of passively transported compounds across the Caco-2 cells (12). If, however, the compound is absorbed by a carrier-mediated mechanism, correlations are often spurious. This could be related to differences in the expression of genes between Caco-2 cells and intestines that are involved in drug uptake, such as transporters and ion channels. Moreover, Caco-2 cells express different phenotypes, i.e., resembling colon crypt cells, small intestinal absorptive enterocytes, and follicle-associated epithelium enterocytes (13); therefore, differentiation status and passage number could strongly affect expression of transporters and ion channels.

For a majority of drugs, it remains unknown which transporters play a role in their absorption and targeting in the body. The human genome project has made it possible to identify most if not all genes encoding transporters and ion channels. This provokes the challenge to determine which transporters interact with any given drug at a genomic scale. A first step in identifying genes relevant to drug absorption in the intestines is to obtain a catalog of all expressed mRNAs. In this study, we used a custom-designed oligonucleotide array to determine the mRNA expression profiles of transporters and ion channels in Caco-2 cells at different passage number and differentiation state, in comparison with mRNA profiles in human intestines. Also, we compared mRNA profiles from small intestine and colon to assess the feasibility of targeting carrier-mediated drugs via different segments in the intestine. The results will be useful in understanding intestinal drug absorption at the molecular level.

MATERIALS AND METHODS

Materials

FastTrack Kit 2.0 and tRNA were obtained from Invitrogen (Carlsbad, CA, USA). Media for cell culture were

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from Cell Culture Facility at UCSF (San Francisco, CA, USA). Material for coating of slides, succinic anhydride and poly(A) + RNA was purchased from Sigma (St. Louis, MO, USA), Gold Seal Microslides from Fisher Scientific (Tustin, CA, USA), and Fluorolink Cy5 Monofunctional Dye 5 and Fluorolink Cy3 Monofunctional Dye 3 from Amersham (Piscataway, NJ, USA). Total RNA from colon (Lot# 1050229, pool from two healthy male Caucasians, age 35 and 50 years) and small intestine (Lot# 1070366, pool from five healthy male and female Caucasians, age between 20 and 65 years) was obtained from Clontech (Palo Alto, CA, USA). Extraction of total RNA was performed by phenol extraction according to a company internal protocol. Oligomers were ordered from MWG (High Point, NC, USA).

Cell Culture

Caco-2 cells passages 81 to 98 were cultured in Dulbecco's modified Eagle's medium with 4.5 g/L of D-glucose), which was supplemented with 20% fetal bovine serum, nonessential amino acids (1% v/v), and 100 units/mL penicillin and 100 μg/mL streptomycin. Confluent monolayers were subcultured every 7 days by treatment with 0.5% trypsin and 0.2% EDTA and seeding at a density of 5×10^5 cells/80 cm^2 into plastic flasks. Cultures were incubated at 37°C in a humidified atmosphere of 5%/95% CO_2/O_2. For mRNA expression measurements in flasks, cells were seeded at a density of 1.5×10^6 cells/175 cm^2, for mRNA expression studies in filters, at a density of 3.75×10^5 cells/44 cm^2 on polycarbonate inserts (Costar Transwell, mean pore diameter of 0.4 μm). The transepithelial electrical resistance (TEER) was measured with a Millicell-ERS (Millipore, Corp., Bedford, MA, USA).

Selection of Genes

Hidden Markov Models (HMMs) of transporter and ion channel genes were selected by searching the Pfam Database 6.1 (http://pfam.wustl.edu/) with keywords and seed sequences chosen from known transporter and channel families (http://www-biology.ucsd.edu/~msaier/transport/toc.html). HMMs were run against the Genpept database using hmmsearch/hmmer-2.1.1-intel-linux (http://hmmer.wustl.edu/). Only hits with a probability of 0.0001 or lower were selected. Using the multiple alignment program ClustalW, redundant accession numbers were filtered out. In addition, new putative transporter and channel gene sequences were collected. An automated search method was applied that uses converged PSI-Blast against the human expressed sequence tags (EST) (1) database for identification of new gene candidates (14). Resulting contig sequences representing two or more overlapping ESTs were used for the array. Because this work was performed before the release of the human sequence, contigs identified in our search were then run against the human genome database, and annotated genes matching the contigs were identified. Several contigs did not match any annotated genes and, therefore, might represent yet uncharacterized genes. Identity of these putative genes will be studied separately. Housekeeping genes and negative controls were the same as in the Atlas 1.2 Human Array by Clontech.

Design of Oligomers

Coding region sequences only were used for the design of the oligomers. To select the 70mers, an algorithm was applied that takes the following four criteria into account: uniqueness, internal palindrome structure (reverse Smith-Waterman algorithm is used to detect palindrome sequence), melting temperature, and localization of the 70mer probe within the gene sequence (15). For the design of the 70mers, a melting temperature of 70°C, an internal palindrome structure value of 100, and a uniqueness cutoff of 15 bp were chosen. All oligomers were designed to be located as close toward the 3′ end as possible. A list of all genes on the chip, including sequences of the 70mers, will be published on AAPS PharmSci, (URL to be provided.)

Preparation of Microarray Chips

Glass slides were coated according to DeRisi et al. (16). Oligomers were printed at a concentration of 33 μM in 3 × SSC. The entire set of 750 oligos was printed four times on each glass slide. After printing, slides were treated with succinic anhydride before hybridization to block poly-L-lysine (16).

Preparation of Poly(A) + RNA, Labeling, Hybridization, and Scanning

Poly(A) + RNA was extracted from cells using the FastTrack 2.0 Kit by Invitrogen. Samples of 2 μg poly(A) + RNA or 20 μg total RNA, respectively, were labeled with either Cy3 Dye or Cy5 Dye by amino-allyl coupling (http://derisilab.ucsf.edu/pdfs/amino-allyl-protocol.pdf,), resuspended in 20 μL of HEPES buffer (25 mM, pH 7.0) containing 1 μL of tRNA, 1.5 μL of yeast poly(A)$^+$ RNA, 0.45 μL of 10% SDS, and hybridized to the slides for 16 h at 65°C. For all hybridization reactions comparing Caco-2 cells poly(A) + RNA was used, whereas for hybridization reactions with human tissue samples total RNA was used. Cells grown in flasks had passage numbers between 81 and 82. For studies in filters, two sets of experiments were done. In the first set, cells had passage numbers between 82 and 98 and in the second set between 82 and 85. Slides were washed and dried before scanning according to DeRisi et al. (16). Slides were scanned on a GenePix 4000A (Foster City, CA, USA) to detect Cy3 and Cy5 fluorescence.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Single-stranded DNA was prepared from Caco-2 total RNA using oligo-dT priming (Thermoscript RT Kit, Invitrogen, Carlsbad, CA, USA), and the same batch of ssDNA was used in all subsequent mRNA-profiling reactions. RT-PCR was performed using the MJ Research Opticon and SYBR-green detection (Applied Biosystems, Foster City, CA, USA). The threshold cycle for PCR products was defined as the cycle at which the SYBR green fluorescent signal was 20 standard deviations above background fluorescence. The threshold cycle for beta-actin and GAPDH mRNA was used to normalize ssDNA for comparison between samples. Calibrations were performed using known concentrations of plasmid cDNA or amplified PCR product. PCR products were checked for appropriate size by gel electrophoresis and were sequenced to verify specificity. For routine studies, melt peak analysis was performed at the end of quantitative PCR experiment to evaluate amplification of the correct product. In

all, ~60 mRNAs encoding transporters were analyzed by RT-PCR. A full report on these data will be provided separately. Sequences of the PCR primers are available upon request.

Analysis of Data

Background subtraction and calculation of average of medians was conducted using GenePix Software 3.0 (Foster City, CA, USA). Cut-off values for selecting differentially expressed genes were based on control studies, i.e., hybridization of identical, but differently labeled poly(A+) RNA. For hybridization studies with combined RNA sources, i.e., total RNA and ploly (A) + RNA, more stringent cut-off values were applied. K-means clustering was done using Spotfire software (Somerville, MA, USA).

RESULTS

Selection of Transporter and Ion Channel Genes

Seventy-nine HMMs were run against the GenePept DB representing various gene families encoding transporters, ion exchangers, and ion channels. The resulting gene sequences were compared with an existing transporter and ion channel database (http://www.aapspharmsci.org/scientificjournals/pharmsci/journal/20.html). A total 670 transporter genes and 261 channel genes (including splice variants) were collected. The search for putative new genes by using a converged PSI-Blast against the human EST database (14) resulted in 236 contigs that differed from known genes. For some gene sequences, 70mer probes could not be designed under the specified conditions. These were either sequences containing single nucleotide polymorphisms or splice variants of known genes. In these cases, a probe sequence was chosen that represented all splice variants. In the case of single nucleotide polymorphisms an oligomer was designed that did not contain the SNP site. After eliminating duplicated sequences, 316 oligomers were designed for transporter genes, 151 for channel genes, 156 for contigs representing putative transporter genes not in the nr database at the time (~January 2001), and 12 for controls. In addition to the above-mentioned genes, the array contained 100 genes representing RGS, ADAM, and EGF-like gene families. These are not further considered in the present study.

Passage Number and Differentiation in Caco-2 Cells

Caco-2 cells were grown over a period of 3 weeks. Under the conditions used, the cells were confluent after 1 week when grown in flasks. When cultured on filters, the TEERs increased between 5 days and 2 weeks and remained stable between 2 weeks and 3 weeks, indicating that cells were differentiated after 2 weeks. For all studies in flasks, Caco-2 cells with passage numbers between 81 and 83 were used, whereas for studies on filters, cells with passage numbers ranging from 82 to 98 were used. To test the error associated with measuring relative mRNA levels between two samples, we compared two identical poly(A) + samples obtained from Caco-2 cells cultured for 5 days, labeled with Cy3 or with Cy5. The mean intensity values for Cy3 and Cy5 fluorescence, from four observations for each probe (spotted four times on a single slide), were plotted as shown in Fig. 1. The scatter along the diagonal is acceptable, and there are no outliers. Deviations

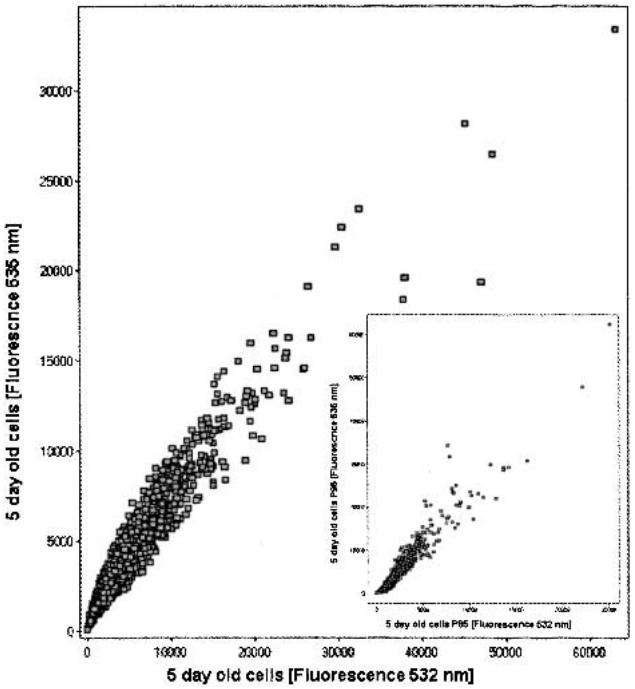

Fig. 1. Comparison of flourescence intensities of identical mRNA samples from 5-day Caco-2 cells passage 82 grown in flasks, labeled with Cy3 or Cy5, respectively. Scatter represents the analytical error. Insert shows comparison of 5-day Caco-2 cells passage 85 vs. 95.

from the line of unity were 1.5-fold maximally. Using three replicate slides, or a total of 12 observations, a change of 1.3- to 1.5-fold in mRNA expression level over the control can be reliably measured, depending upon the probe and fluorescence intensity of the signal. Thus, we used as a cut-off 0.45 for the $\log_2$ ratio values, unless noted otherwise.

To assess the influence of passage number on the mRNA expression profile, we compared cells that were grown on filters with passage numbers 85 and 98 (cf. Fig. 1, insert). Table I shows all genes that were significantly up- or down-regulated. *GLUT3* was the only gene that showed a 2-fold change in mRNA expression between the different passage numbers. A few genes displayed a change in mRNA expression of 1.5-fold or higher, indicating that the cellular phenotype does change detectably with passage number.

The next series of experiments addressed the question how the mRNA expression profile changes with differentiation of Caco-2 cells. Poly(A) + RNAs of cells cultured for 5 days, 1 week, 2 weeks, and 3 weeks were fluorescently labeled and their mRNA expression profiles analyzed. Over a period of 3 weeks, the mRNA expression profile changed markedly. Figures 1–4 show the fluorescence intensity of Cy3 labeled mRNA of 5-day-old cells (Cy3) vs. the fluorescence of Cy5-labeled mRNA of 1-, 2-, and 3-week-old cells. Changes occur mainly between 5 days and 1 week and between 1 week and 2 weeks (cf. Fig. 5). Tables II and III show all genes that are significantly up- or down-regulated over a period of 3 weeks. Only a few genes had a 2-fold or higher change in mRNA expression levels measured by the ratio value. There were no major differences observed between cells grown on filters or in plastic flasks. All genes that had a 2-fold or higher change in mRNA expression ratio in cells grown on flasks were similarly regulated in cells grown on filters. Figure 6 shows the

Table I. Genes Showing Significant Changes in mRNA Expression with Increasing Passage Numbers in Caco-2 Cells

Name	ID	Log ratio values	SD
ABCG5	gil11692799	−0.60	0.48
ACCN3	gil9998947	0.66	0.22
Bak-3 pseudogene	gil595923	0.75	0.10
Ca-act. Cl channel protein	gil5726286	−0.87	0.87
Connexin 59 CX59	gil9957541	−0.68	0.74
Contig101 = protein similar to AQP7	gil9367917	−0.89	0.82
Contig103 = AQP7	gil4502186	−0.61	0.50
Contig14 = ABCA5	gil20559208	−0.64	0.52
Contig17 = HepG2/SLC2A1	gil183302	0.62	0.47
Contig171 = cofactor required for Sp1	gil13937750	−0.63	0.54
Contig223 = FLJ23784	gil18676946	−0.84	0.60
Contig81 = SLC7A8, CAT, y+ system	gil14751202	0.61	0.33
Electrogenic Na+ bicarbonate cotrans.	gil4877551	−0.63	0.63
GLUT3	gil183684	1.26	0.19
LTRPC7	gil13959784	−0.60	0.37
SLC12A3: Na/Cl transporters	gil4506976	0.71	0.36
SLC21A14: organic anion transp. polypeptide 14	gil7839586	−0.81	0.66
SLC30A3, zinc transporter	gil4508042	0.70	0.21
Stromelysin	gil36632	0.74	0.43
TIRC7	gil3603174	0.63	0.37

Note: Relative mRNA expression levels are $\log_2$ ratio values of Cy5 intensities in P95 cells over Cy3 intensities in P85 cells. Cut-off for $\log_2$ ratio values was 0.6 ($n = 2 \times 4$).

K-means clustering of relative mRNA expression levels in flask and filters. This groups genes together that have a similar mRNA expression profile over the time course of differentiation. Clusters 8 to 12 show genes that are more dynamically regulated over time than genes represented in clusters 1 to 7. Clusters 8 and 12 contain genes that show a trend to down regulation upon differentiation, whereas clusters 10 and 11 contain genes with a trend to up regulation. In summary, transporter genes that that are up-regulated upon differentiation include *GLUT5,* amino acid and ion transporters, such as *Nramp2, MAT8,* and *Na^+,K^+-ATPase,* whereas amino acid transporter *E16* and the glucose transporter *GLUT3* were

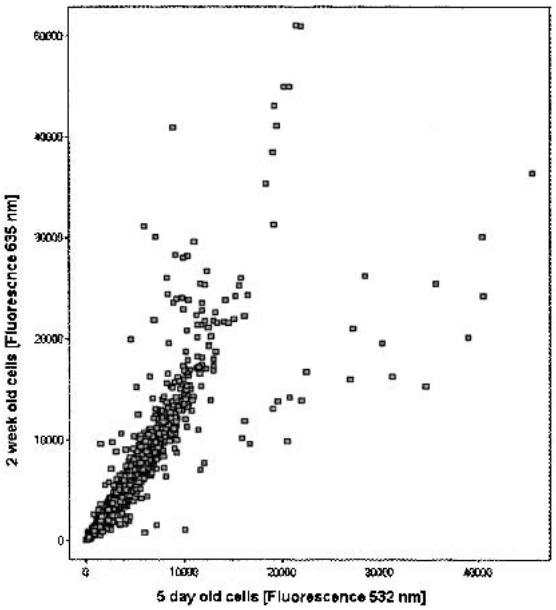

Fig. 3. Comparison of flourescence intensities of mRNA samples from 5-day (P82) and 2-week Caco-2 cell cultures (P81) grown in flasks, labeled with Cy3 or Cy5, respectively.

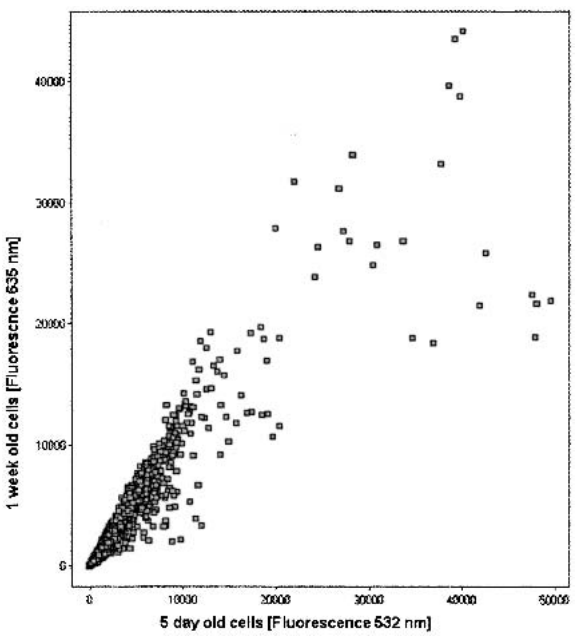

Fig. 2. Comparison of flourescence intensities of mRNA samples from 5-day and 1-week Caco-2 cell cultures (all passage P82) grown in flasks, labeled with Cy3 or Cy5, respectively.

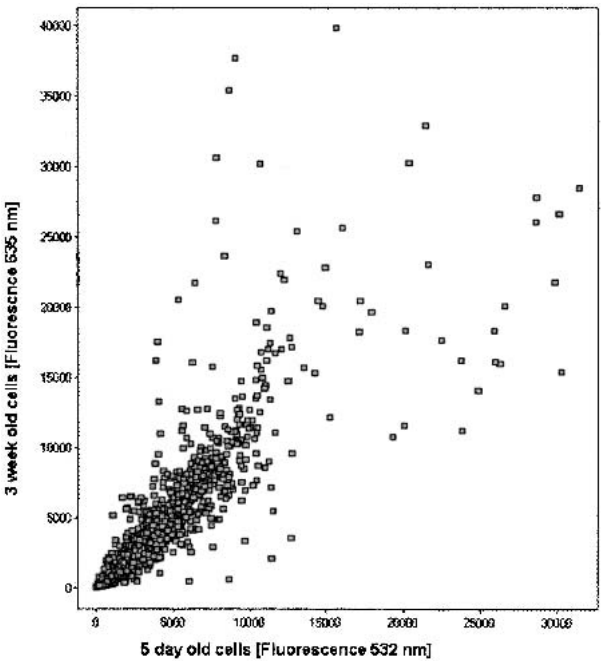

Fig. 4. Comparison of flourescence intensities of mRNA samples from 5-day (P82) and 3-week (P82) Caco-2 cell cultures grown in flasks, labeled with Cy3 or Cy5, respectively.

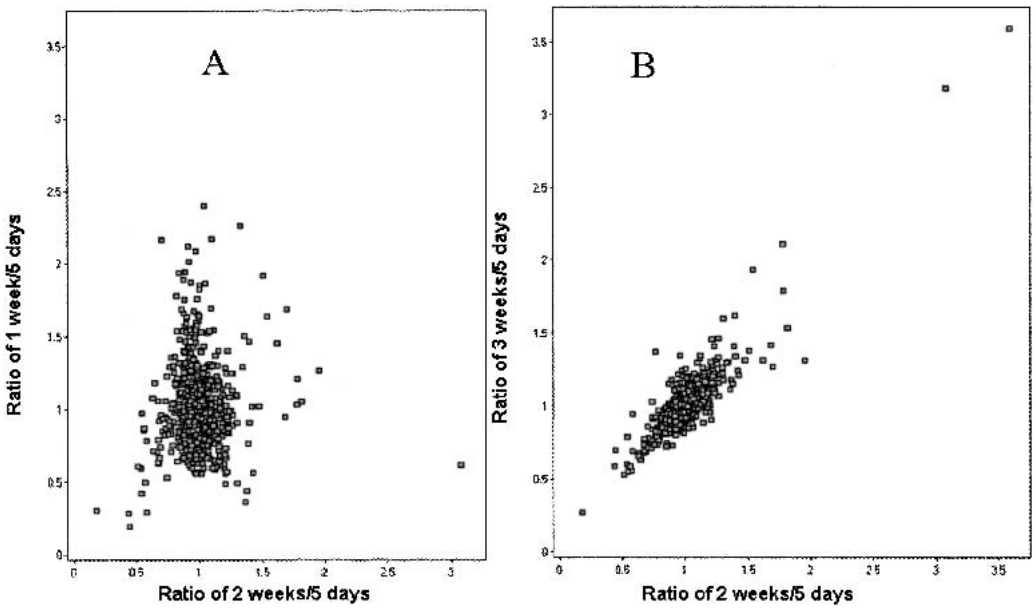

Fig. 5. Plot of relative mRNA expression levels between 2-weeks and 1-week Caco-2 cell cultures and between 2-week and 3-week cell cultures. Points along the diagonal indicate little change in expression ratio between 2- and 3-week cultures.

significantly down-regulated. In addition, genes of the ABC superfamily were regulated upon differentiation. The array contains probes for 40 of the currently known 48 ABC transporter genes—an indication of the overall coverage of the probe array for a well-defined transporter family (14). No influence of the state of differentiation was observed for the dipeptide transporter hPept1 and the monocarboxylic acid transporters—that is, ratio values were smaller than ~1.2, i.e, log2 ratio values were all lower or equal to ~0.26). This result is surprising because hPepT1 is thought to be prominently up-regulated in differentiated Caco-2 cells (17). Other factors, including protein expression at the cell surface rather than at intracellular membranes, may play a role in the enhanced drug/dipeptide transport in differentiated Caco-2 cells.

Quantitative RT-PCR

To validate the results obtained with array, we also measured mRNA expression by RT-PCR, an accepted quantitative method. With quantitative RT-PCR, an up-regulation of hPept1 was observed in Caco-2 cells upon differentiation over 2 weeks. In general, RT-PCR was more sensitive in detecting changes of gene expression during differentiation than the microarray. All genes with a 5-fold or higher increase over 2 weeks (*ABCC2, ABCG1, OCTN1, RBAT, ABCA1, ABCA5, ABCC3, GLUT5, SLC5A3, NPT-4, DRA*) using RT-PCR were also found to be up-regulated in both flasks and filters (except *NADC1*) using the microarray, but with an average $\log_2$ value of only 0.22 in filters and 0.36 in flasks. However, genes with a $\log_2$ value of 0.45 or higher in microarrays (*ABCC1, ABCA5, ABCC3, GLUT5, SLC5A3, OATPRP2*) were always confirmed with RT-PCR (Table IV) Moreover, we compared the mRNA expression of 22 genes (OAT1, MCT1, MCT3, CAT2, and 18 ABC transporters) that did not change significantly during 2-week differentiation measured by RT-PCR (2-fold or lower with RT-PCR), to results obtained with microarray data. Consistent with the RT-PCR data, the average $\log_2$ value was 0.04 using microarrays, indicating no significant change. With RT-PCR, absolute levels of the mRNAs can be estimated, whereas the array only detects changes in mRNA expression (because signal intensity is subject to many variables).

Comparison between Caco-2 Cells and Intestinal Tissue

The mRNA expression of transporter and ion channel genes in undifferentiated 5-day-old and differentiated 3-week-old Caco-2 cells were compared with mRNA from human intestinal tissue. Differences between Caco-2 cells and tissue were more pronounced than between undifferentiated and differentiated Caco-2 cells. The mRNA expression of numerous genes differed by 2-fold or more. Genes that are known to be specifically expressed in defined segments in the intestine were found to be more highly expressed relative to Caco-2 (cf. Tables V and VI), such as *SCNN1B* (18) and colon mucosa-associated *DRA* (19) in the colon, and *SGLT1* (20) and CaCC1 (21) in the small intestine. Confirmation of high mRNA levels for these intestinal tissue-selective genes validates further the results obtained with the custom-design array. As expected, *GLUT3* mRNA was at low abundance in colon and small intestine (22). The mRNA expression of several genes that were significantly regulated in Caco-2 cells upon differentiation was similar between human intestines and differentiated Caco-2 cells. For example, *MAT8,* was strongly up-regulated upon differentiation, reaching a similar level of mRNA expression as found in colon tissue. In contrast, *MAT8* was expressed to similar levels in undifferentiated Caco-2 cells and small intestine. Other examples that showed a similar pattern are S-protein, sulfate transporter *DTD,* and *Na,K-ATPase b1* gene. No members of the ABC transporters, the dipeptide transporters, and monocarboxylic acid transporters had a log ratio value of one or higher. How-

Table IIA. Genes That Are Regulated in Caco-2 Cells Grown in Flasks over a Period of 3 Weeks

Name (K-means cluster)[a]	ID	Log ratio values (1 week)	SD	Name (K-means cluster)[a]	ID	Log ratio values (2 weeks)	SD	Name (K-means cluster)[a]	ID	Log ratio values (3 weeks)	SD
AAT E16 (12)	gi|3639057	–0.46	0.50	AAT E16 (12)	gi|3639057	–2.08	0.82	AAT E16 (12)	gi|3639057	–0.90	0.32
				ATA1 (8)	gi|11640742	–0.70	0.59	ATA1 (8)	gi|11640742	–0.91	0.64
				ABC-1 (5)	gi|4128032	0.61	0.61	ABC-1 (5)	gi|4128032	0.61	0.48
				ABCC3 (6)	gi|9955969	0.54	0.58	ABCC3 (6)	gi|9955969	0.47	0.56
				Contig8 = ABCA5 (4)	gi|20559208	0.46	0.41	Contig8 = ABCA5 (4)	gi|20559208	0.52	0.22
				GLUT3 (9)	gi|183684	–0.72	0.24	GLUT3 (9)	gi|183684	–0.76	0.52
				Importin alpha 3 (8)	gi|1928974	–0.58	0.50	Importin alpha 3 (8)	gi|1928974	–0.73	0.62
MAT8 (11)	gi|1085025	–0.49	0.47	MAT8 (11)	gi|1085025	1.42	0.51	MAT8 (11)	gi|1085025	1.85	0.42
				Na,K-ATPase b1 (10)	gi|806753	0.86	0.23	Na,K-ATPase b1 (10)	gi|806753	0.67	0.80
				NRAMP2 (10)	gi|3869152	0.68	0.32	NRAMP2 (10)	gi|3869152	1.10	0.40
				PMP70 (9)	gi|35552	–0.70	0.54	PMP70 (8)	gi|35552	–0.91	0.60
				RCH1 (8)	gi|791184	–0.56	0.36	RCH1 (8)	gi|791184	–0.60	0.43
				S-protein (11)	gi|36574	1.38	0.34	S-protein (11)	gi|36574	1.69	0.41
SUR2 (8)	gi|3127138	–0.48	0.43	SUR2 (8)	gi|3127138	–0.50	0.52	SUR2 (8)	gi|3127138	–0.55	0.68
				Tim17a (8)	gi|4378528	–0.56	0.61	Tim17a (8)	gi|4378528	–0.65	0.94
				TIM23 (8)	gi|2599128	–0.67	0.54	TIM23 (8)	gi|2599128	–0.84	0.66

Note: Relative expression levels are $\log_2$ ratio values of Cy5 intensities in 1-, 2-, and 3-week-old cells over Cy3 intensities in 5-day-old cells. Cut-off for $\log_2$ ratio values was 0.45 ($n = 4 \times 3$).
[a] K-means cluster number according to Fig. 6.

Table IIB. Genes That Are Regulated in Caco-2 Cells Grown in Flasks over a Period of 3 Weeks

Name (K-means cluster)[a]	ID	Log ratio values (1 week)	SD	Name (K-means cluster)[a]	ID	Log ratio values (2 weeks)	SD	Name (K-means cluster)[a]	ID	Log ratio values (3 weeks)	SD
Bcl-xL/bcl-2 ass. death promoter B (5)	gi|2660728	–0.47	0.52	BCL2/adenovirus E1B (1)	gi|558845	0.60	0.44	EAAT1 (8)	gi|487338	–0.67	1.37
CAT, y+ system (1)	gi|7657682	–0.60	0.59	Contig223 = FLJ23784 (7)	gi|18676946	–0.47	0.73	FCGRT (6)	gi|4758345	0.45	0.35
Contig222 = ATP9B (3)	gi|20554286	–0.48	0.39	SLC5A3: Na+/myo-inositol cotrans. (6)	gi|662842	0.46	0.16	HK1 (5)	gi|184100	–0.45	0.46
Contig72 = clone hCIT.281_F_24 (3)	gi|3176728	–0.56	0.45	Sulfate transporter DTD (10)	gi|549987	0.49	0.30	HPT-1 (10)	gi|483391	0.55	0.24
Contig9 = ABCA6 (7)	gi|20559210	–0.46	0.58					OATPRP2 (10)	gi|11990588	0.58	0.33
Creatine transporter (3)	gi|493131	–0.46	0.52					SCN10A (8)	gi|4838144	–0.52	0.27
hASCT1 (3)	gi|765233	–0.45	0.58					SLC17A4: sodium phosphate (4)	gi|4885440	0.63	0.63
Stimulator of Fe transport (7)	gi|2738924	–0.47	0.58					SLC3A2: act. of dibasic and neutral AAT (8)	gi|4505140	–0.51	0.31
SLC21A14: organic anion transp. polypeptide 14 (7)	gi|7839586	–0.52	0.53					SRP1 homolog (5)	gi|913392	–0.52	0.69
SLC21A3: organic anion transporter (4)	gi|4827013	–0.49	0.41								

Note: Relative expression levels are $\log_2$ ratio values of Cy5 intensities in 1-, 2-, and 3-week-old cells over Cy3 intensities in 5-day-old cells. Cut-off for $\log_2$ ratio values was 0.45 ($n = 4 \times 3$). Genes listed here reached the cut-off value at only one time point.

[a] K-means cluster number according to Fig. 6.

Table IIIA. Genes That Are Regulated in Caco-2 Cells Grown in Filters over a Period of 3 Weeks

Name (K-means cluster)[a]	ID	Log ratio values (1 week)	SD	Name (K-means cluster)[a]	ID	Log ratio values (2 weeks)	SD	Name (K-means cluster)[a]	ID	Log-ratio values (3 weeks)	SD
AAT E16 (12)	gi|3639057	−0.54	0.23	AAT E16 (12)	gi|3639057	−0.81	0.71	AAT E16 (12)	gi|3639057	−1.46	0.33
				ATA1 (8)	gi|11640742	−0.48	0.57	ATA1 (8)	gi|11640742	−0.58	0.45
MAT8 (11)	gi|1085025	0.62	0.73	MAT8 (11)	gi|1085025	0.92	0.65	MAT8 (11)	gi|1085025	2.29	0.52
				NRAMP2 (10)	gi|3869152	0.77	0.23	NRAMP2 (10)	gi|3869152	2.24	0.19
				S-Protein (8)	gi|36574	0.52	1.46	S-Protein (8)	gi|36574	1.92	0.40
SLC21A14: organic anion transp. polypeptide 14 (7)	gi|7839586	−0.53	1.07					SLC21A14: organic anion transp. polypeptide 14 (7)	gi|7839586	−0.50	0.43

Note: Relative expression levels are $\log_2$ ratio values of Cy5 intensities in 1-, 2-, and 3-week-old cells over Cy3 intensities in 5-day-old cells. Cut-off for $\log_2$ ratio values was 0.45 ($n = 4 \times 3$).
[a] K-means cluster number according to Fig. 6.

Table IIIB. Genes That Are Regulated in Caco-2 Cells Grown in Filters over a Period of 3 Weeks

Name (K-means cluster)[a]	ID	Log ratio values (2 weeks)	SD	Name (K-means cluster)[a]	ID	Log ratio values (3 weeks)	SD
BCL2/adenovirus E1B 19kD-interacting protein 1)	gi|558845	0.53	0.35	Contig101 = novel gene similar to AQP7 (7)	gi|9367917	−0.57	0.48
Contig223 = FLJ23784 (7)	gi|18676946	−0.55	0.55	Contig103 = AQP7 (7)	gi|4502186	−0.60	0.56
SLC12A2: Na/K/Cl transporters (8)	gi|4506974	−0.55	0.71	EAAT1 (8)	gi|487338	−0.69	0.65
				FCGRT (6)	gi|4758345	0.74	0.54
				GLUT3 (9)	gi|183684	0.64	0.46
				GLUT5 (6)	gi|183297	1.10	0.32
				HepG2 (2)	gi|183302	0.76	0.36
				HPT-1 (10)	gi|483391	1.19	0.14
				Importin alpha 3 (8)	gi|1928974	−0.45	0.28
				Na,K-ATPase b-1 (10)	gi|806753	1.82	0.22
				OATPRP2 (10)	gi|11990588	1.23	0.21
				PMP70 (8)	gi|35552	−0.72	0.27
				RCH1 (8)	gi|791184	−0.69	0.15
				Sulfate transporter DTD (10)	gi|549987	1.64	0.41
				SUR2 (8)	gi|3127138	−0.51	0.32
				Tim17a (8)	gi|4378528	−0.54	0.27

Note: Relative expression levels are $\log_2$ ratio values of Cy5 intensities in 1-, 2-, and 3-week-old cells over Cy3 intensities in 5-day-old cells. Cut-off for $\log_2$ ratio values was 0.45 ($n = 4 \times 3$). Genes listed here reached the cut-off value at only one time point.
[a] K-means cluster number according to Fig. 6.

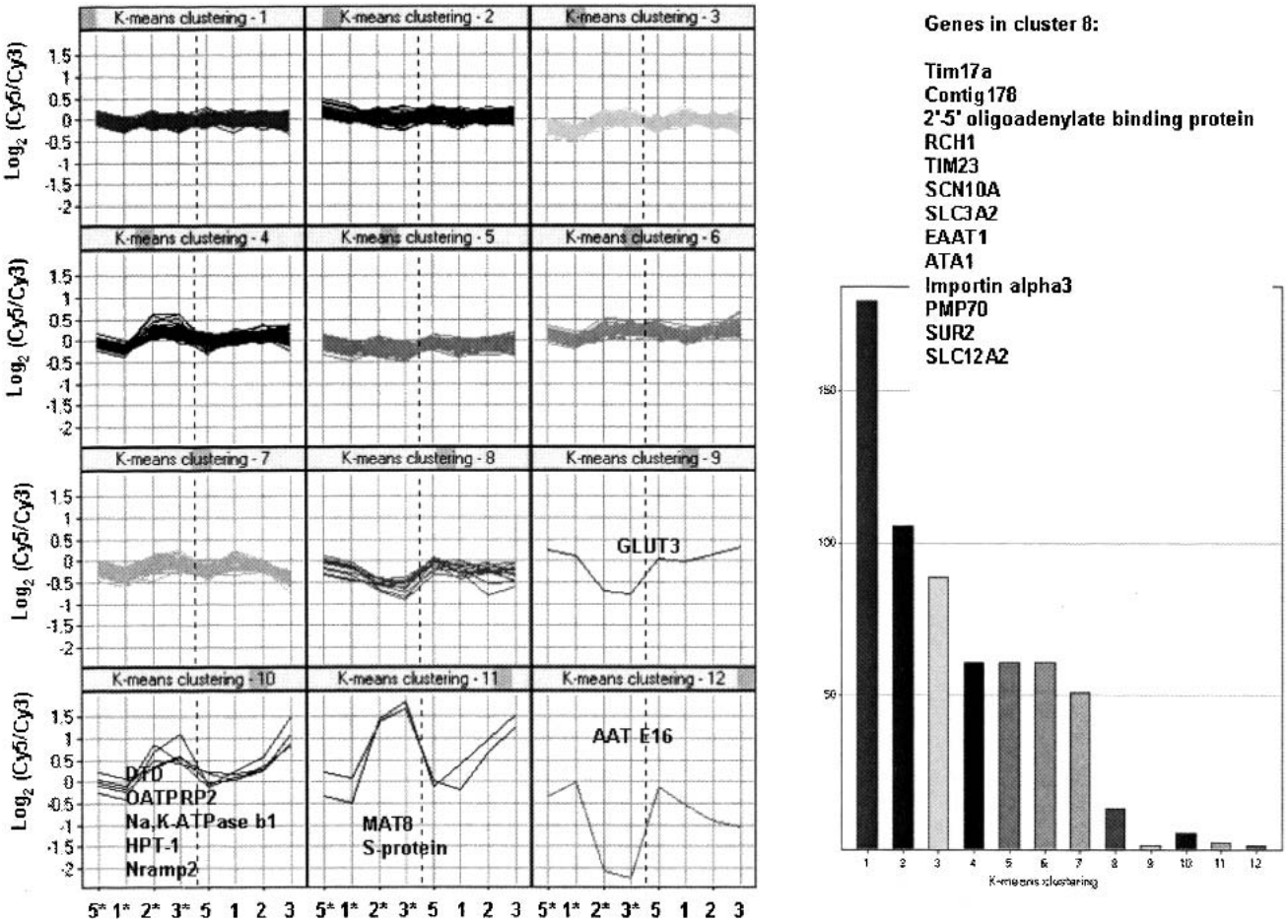

Fig. 6. K-means clustering of relative mRNA expression [$\log_2$ (Cy5/Cy3)] levels after 5-day, 1-, 2-, and 3-week cultures in flasks (5*, 1*, 2*, 3*) and on filters (5, 1, 2, 3). Similarity measure: Euclidean distance. Cluster initialization: Data centroid based search. Bar chart shows the number of genes per each cluster.

ever, this cut off value is rather stringent as we can detect smaller changes using four replicate samples per array and three separate experiments. Table VII lists all members of these families that were differently regulated in 3-week-old Caco-2 cells compared with intestinal tissue assuming that a cut-off value of 0.45 is significant. This includes hPepT1, which is selectively expressed in small intestines.

Table IV. Comparison of RT-PCR and Microarray

Name	ID	Microarray: log ratio value	SD	RT-PCR: log ratio values
OATPRP2[a]	gi|11990588	1.23	0.21	2.40
GLUT5[a]	gi|183297	1.10	0.32	6.91
ABC-1	gi|4128032	0.61	0.48	5.01
OATPRP2	gi|11990588	0.58	0.33	2.40
ABCA5	gi|20559208	0.52	0.22	2.17
ABCC3	gi|9955969	0.47	0.56	8.29
SLC5A3[b]	gi|662842	0.46	0.16	3.53

Note: Listed are genes with a $\log_2$ ratio value of 0.45 or higher using microarray. Relative mRNA expression levels are $\log_2$ ratio values of Cy5/Cy3 intensities for 3-week vs. 5-day-cell cultures.
[a] On filters.
[b] After 2 weeks.

Comparison between Colon and Small Intestine

Genes that were found earlier (Tables V and VI) to be specific for either the colon or the small intestine in comparison to differentiated Caco-2 cells were confirmed by direct comparison of mRNAs from small intestine with colon (cf. Table VIII). For instance *CaCC2,* contig90, a gene similar to *CAT, y* + system (gi|20485816), and *MAT8* were found to be more highly expressed in colon, and *GLUT5* and *SGLT1* in small intestine. By lowering the cut-off value from 0.6 to 0.45, only three additional members of the ABC transporter, and the dipeptide transporter *hPepT1* were found to be differently expressed in small intestines and colon: *ABCG8* (–0.60 ± 0.45), *ABCB8* (0.41 ± 0.33), and *hPept1* (–0.56 ± 0.24).

DISCUSSION

The importance of carrier-mediated transport on drug bioavailability remains to be assessed for many drugs. The large number of transporter genes in the human genome suggests that transport mechanisms may play a pervasive role in drug absorption and tissue targeting. Moreover, ATPases and ion channels determine the electrochemical gradient across the plasma membrane and generate the driving force for pri-

Table V. Comparison of Genes That Are Differently Regulated in Caco-2 Cells and Colon Tissue

Name	ID	Log ratio values (5-day)	SD	Name	ID	Log ratio values (3-week)	SD
CaCCl	gi|4585468	1.83	0.69	CaCC1	gi|4585468	1.90	0.63
CLNS1A	gi|717053	–1.91	0.65	CLNS1A	gi|717053	–1.61	0.66
Colon mucosa-associated DRA	gi|291963	2.71	0.67	Colon mucosa-associated DRA	gi|291963	1.86	0.44
Contig90 = similar to CAT, y+ system	gi|20485816	1.85	0.49	Contig90 = similar to CAT, y+ system	gi|20485816	2.41	0.39
GLUT3	gi|183684	–3.56	0.65	GLUT3	gi|183684	–2.51	1.25
NBC4	gi|10864004	1.52	0.13	NBC4	gi|10864004	1.74	0.39
NHE-1	gi|544775	1.55	0.13	NHE-1	gi|544775	1.88	0.21
SCNN1B	gi|1004270	2.65	0.32	SCNN1B	gi|1004270	3.01	0.35
SLC7A7: CAT, y+ system	gi|4507054	–1.57	1.19	SLC7A7: CAT, y+ system	gi|4507054	–1.73	1.40
S-protein	gi|36574	–2.90	1.35	S-protein	gi|36574	–4.78	1.55
AAT E16	gi|3639057	–2.63	0.94	Contig226	Contig226	1.70	0.28
EAAT1	gi|487338	–1.56	1.00	Contig81	Contig81	1.55	0.21
MAT8	gi|1085025	1.61	0.37	EAAT5	gi|2076761	1.82	0.35
Sulfate transporter DTD	gi|549987	1.97	0.28	HepG2	gi|183302	–1.60	1.19
				KCNE1	gi|4557686	1.88	0.35
				NRAMP2	gi|3869152	–2.49	0.71
				P2RX1	gi|4505544	1.61	0.34
				Potassium ionic channel Kv4.3	gi|6644149	1.53	0.14

Note: Relative mRNA expression levels are $\log_2$ ratio values of Cy5 intensities in colon tissue over Cy3 intensities in 5-day-old or 3-week-old cells. Cut-off for $\log_2$ ratio values was 1 ($n = 4 \times 3$).

mary and secondary active transport. The present study provides a survey of those genes that are expressed in Caco-2 cells and in human intestines, as a first step in understanding the overall transport capacity of human intestinal tissue relevant to drug bioavailability, and the utility of Caco-2 cell culture as a model of intestinal drug absorption. Microarray technology allows one to measure mRNA expression levels of a large number of genes. We used two methods for identifying gene targets for our chip: 1) screening the NCBI database for ESTs that are putative members of transporter/channel families (14) and 2) screening the Genepept database with HMMs that represent transporter and channel genes. This strategy allowed us to design a chip that contained genes with known function, putative transporter/channel genes with known open reading frame, and new putative genes not represented in NCBI as open reading frames at the time. Upon completion of the study, we have checked all contigs—assembled from overlapping ESTs—against the nr database of NCBI, and have identified those genes that have since been annotated. The newly listed genes represented by contigs are indicated in the tables. For example, among the 48 known ABC transporter genes, we had probes for 32 genes listed at the time and for eight contigs representing genes listed subsequently. The coverage of our probes may vary from one gene

Table VI. Comparison of Genes That Are Differently Regulated in Caco-2 Cells and Small Intestinal Tissue

Name	ID	Log ratio values (5-day)	SD	Name	ID	Log ratio values (3-week)	SD
CaCCl	gi|4585468	2.59	0.98	CaCC1	gi|4585468	1.91	0.89
GLUT3	gi|183684	–3.75	0.74	GLUT3	gi|183684	–2.84	1.05
S-protein	gi|36574	–3.70	1.07	S-protein	gi|36574	–4.13	1.48
AAT E16	gi|3639057	–2.13	0.43	CLNS1A	gi|717053	–1.95	0.87
ATA1	gi|11640742	–1.58	0.54	MAT8	gi|1085025	–2.85	0.74
C1 channel regulatory protein	gi|717053	–2.13	0.45	Na,K-ATPase b1	gi|806753	–1.86	0.58
EAAT1	gi|487338	–1.82	0.98	NRAMP2	gi|3869152	–2.40	0.63
HepG2	gi|183302	–1.63	1.08				
Karyopherin alpha 3	gi|2190277	–1.96	0.42				
RCH1	gi|791184	–1.84	0.51				
SGLT1	gi|338054	1.99	0.77				

Note: Relative expression levels are $\log_2$ ratio values of Cy5 intensities in small intestinal tissue over Cy3 intensities in 5-day-old or 3-week-old cells. Cut-off for $\log_2$ ratio values was 1 ($n = 4 \times 3$).

Table VII. Comparison of Genes of the ABC Transporter, the Monocarboxylic Acid Transporter, and the Dipeptide Transporter Families That Are Differently Regulated in Caco-2 Cells and Colon and Small Intestinal Tissue

Name	ID	Log ratio values colon	SD	Name	ID	Log ratio values small intestine	SD
ABC-1	gi|4128032	–0.47	0.61	ABC-1	gi|4128032	–0.60	1.01
Contig 13 = ABCA10	gi|18587266	0.53	0.22	Contig13 = ABCA10	gi|18587266	0.54	0.25
ABCB1	gi|4505768	–0.89	0.51	ABCB1	gi|4505768	–0.81	0.68
ABCB10	gi|9961243	–0.58	0.57	ABCB10	gi|9961243	–0.62	0.53
ABCB6	gi|9955962	0.63	0.15				
ABCB7	gi|9665249	–0.79	0.49	ABCB7	gi|9665249	–0.96	0.68
ABCB8	gi|9955964	1.12	0.12	ABCB8	gi|9955964	0.61	0.22
ABCC2	gi|4557480	–0.60	0.37	ABCC2	gi|4557480	–0.65	0.39
ABCD4	gi|4826957	–0.70	0.58				
ABCG2	gi|4757849	–0.76	0.48	ABCG2	gi|4757849	–0.72	0.28
				ABCG5	gi|11692799	0.61	0.37
ABCG8	gi|11692801	1.12	0.55	ABCG8	gi|11692801	1.16	0.32
				CFTR	gi|180331	–1.13	0.53
				PMP70	gi|35552	–0.67	0.58
hPept1	gi|773587	–0.86	0.87				
MCT2	gi|3834394	–0.60	0.41				
RF-hPept1	gi|2506042	0.69	0.38	RF-hPept1	gi|2506042	0.53	0.24
SLC16A4	gi|4759113	–1.16	0.89	SLC16A4	gi|4759113	–0.75	0.88
SUR2	gi|3127138	–0.69	0.62				

Note: Relative expression levels are $\log_2$ ratio values of Cy5 intensities in colon or small intestinal tissue over Cy3 intensities in 3-week-old cells. Cut-off for $\log_2$ ratio values was 0.45 ($n = 4 \times 3$).

family to another, but in the case of the ABC transporters, it is 40 of 48 known genes. However, additional genes may exist that have not yet been annotated; in our search of the human EST database (14) we have identified numerous EST singletons that could not be assembled into contigs. These could represent unknown transporter genes, but EST singletons were not included with the microarray.

Many of the genes that are responsible for active transport in the intestine are known to be expressed in Caco-2 cells. Consequently, we investigated the mRNA expression of

Table VIII. Comparison of Small Intestinal and Colon Tissue

Name	ID	Log ratio values	SD	Name	ID	Log ratio values	SD
ABCA2	gi|9957466	0.80	0.20	AQP3	gi|9257193	–0.71	0.28
ABCC3	gi|9955969	0.85	0.51	Citrate transporter	gi|950003	–0.71	0.44
SERCA3b	gi|3004458	0.61	0.30	Contig224 = FATP	gi|16162902	–0.69	0.28
CaCC2	gi|5726288	0.78	0.67	FPN1	gi|7109248	–0.65	0.29
Contig19 = ACATN	gi|6042194	0.64	0.30	GLUT5	gi|183297	–0.90	0.36
Contig26 = HepG2/SLC2A1	gi|5730050	0.62	0.23	NADC1	gi|1098556	–0.66	0.25
Contig90 = similar to CAT, y+ system	gi|20485816	0.83	0.36	SERCA3	gi|2052511	–0.78	0.58
KCNE1	gi|4557686	0.89	0.25	SGLT1	gi|338054	–1.53	0.37
MAT8	gi|1085025	0.80	0.52	SLC20A2: phosphate transporter	gi|5803172	–0.65	0.40
Neurotransmitter transporter, glycine	gi|5902093	0.61	0.35	SLC9A3R1: sodium/hydrogen exchanger	gi|4759139	–0.95	0.29
SCNN1B	gi|1004270	0.83	0.51	Sodium-coupled nucleoside transporter	gi|4759131	–0.66	0.39
SCNN1G	gi|1004272	0.71	0.26				
Na channel, nonvoltage-gated 1 alpha	gi|4506814	0.63	0.43				

Note: Relative expression levels are $\log_2$ ratio values of Cy5 intensities in small intestinal tissue over Cy3 intensities in colon tissue. Cut-off for $\log_2$ ratio values was 0.6 ($n = 4 \times 3$).

transporter and channel genes in Caco-2 cells and compared them with small intestine and colon using microarrays. Caco-2 cells spontaneously convert from undifferentiated cells into polarized cells, requiring the expression of different proteins at the basolateral and apical sides of the cells. Therefore, we investigated the impact of differentiation on gene mRNA expression (23–26) at a genomic level.

Relative mRNA expression levels clearly changed over time. However, the relative increases of mRNA expression levels measured by microarrays were smaller than expected based on quantitative PCR results of some selected genes. This is common to oligonucleotide arrays measuring mRNA levels and may be related to a background level of nonspecific binding under the conditions used. However, genes that were significantly up-regulated, i.e., ~1.4-fold increase or higher, could be confirmed by quantitative PCR (*GLUT5, OATPRP2, ABCC3, ABCA5* [Contig8], *SLC5A3, ABC1*), or were implicated in Caco-2 cell differentiation in previous studies on individual genes. Gene mRNA expression changed during the first 2 weeks and reached a plateau between 2 to 3 weeks, which parallels changes of the TEERs. This pattern correlates with the stage of morphological differentiation (27). As shown earlier, Caco-2 cells differentiate spontaneously after having reached confluency and are fully differentiated after 2 weeks. Only a few genes were strongly regulated over time. Most of these genes encode amino acid transporters, sugar transporters, and ion transporters. *MAT8,* the *S-protein, Nramp2,* and *Na,K-ATPase b1* had the most significant increase in mRNA expression levels. These channels and transporters are either related to chloride and iron homeostasis of the cells or to cell adhesion (28–31). This indicates that the development of an ion gradient and cell adhesion are crucial factors for polarization of the cells.

In human intestines, mRNA expression of a number of genes differed markedly from that in Caco-2 cells, regardless of the state of differentiation. For several genes, e.g., *MAT8, Nramp2, Na,K-ATPase b1, EAAT1,* and *HepG2,* differentiation of Caco-2 cells cause changes in mRNA expression that approximate the mRNA expression found in either small or large intestines. A few genes were more highly expressed in Caco-2 than in intestines: *GLUT3* and the *S-protein. GLUT3* is known to be expressed in Caco-2 cells (32). Earlier studies have demonstrated that *GLUT3* does not appear to be expressed in healthy intestinal tissue, but it was shown to be expressed in various tumors (22,33). Hence, the decrease of *GLUT3* mRNA expression upon differentiation is consistent with a phenotypic change from colon-carcinoma-like into a non-tumor like epithelial cell. The over-expression of *S-protein,* a cell adhesion-promoting plasma and tissue protein, compared to intestinal tissues, and the increased mRNA expression upon differentiation in Caco-2 cells indicate that cell adhesion mechanisms are crucial in Caco-2 cell differentiation. Overall, upon differentiation, Caco-2 cells become more similar to small intestinal cells (Tables V and VI). Many genes were similarly expressed in these two tissues, i.e., their relative mRNA expression values were below the cut-off 0.45 of the log ratio values. Similarly, Engle et al. (13) showed that after reaching confluence, Caco-2 cells expressed proteins characteristic of colonocytes and small intestinal enterocytes. Thereafter, the content of colonocyte-specific proteins decreased, whereas those specific for the enterocytes increased. However, a number of genes appear to be differently expressed in Caco-2 cells compared to the small intestine and colon.

As to members of the ABC transporter, the monocarboxylic acid transporter, and the dipeptides transporter family, well-known transporters that act as carriers of a variety of drugs, we observed significant mRNA expression changes only for a few ABC transporters during differentiation. Under less stringent but still significant criteria, hPept1 was expressed less in the colon than in 3-week-old Caco-2 cells, whereas it was more highly expressed in the small intestine, which confirms earlier studies (17,34). As for the monocarboxylic acid transporters we were unable to observe any significant changes during differentiation, or among the tissues examined. It needs to be kept in mind that the use of mRNA expression arrays—although providing a global picture of the mRNA expression of multiple genes—is less sensitive than other methods such as RT-PCR.

In summary, we have characterized the mRNA expression profiles of numerous genes encoding transporters and ion channels, in differentiating Caco-2 cells and human small intestine and colon. The list of genes and oligonucleotide probes will be available elsewhere (AAPS PharmSci, URL to be provided). Fully differentiated Caco-2 cells can serve as a useful model for studying active drug transport, but the expression of a number of transporters does not appear to reflect mRNA levels in human intestines.

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