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Cell culture modeling of specialized tissue: identification of genes expressed specifically by follicle-associated epithelium of Peyer's patch by expression profiling of Caco-2/Raji co-cultures

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Abstract

Peyer's patch follicle-associated epithelium (FAE) regulates intestinal antigen access to the immune system in part through the action of microfold (M) cells which mediate transcytosis of antigens and microorganisms. Studies on M cells have been limited by the difficulties in isolating purified cells, so we applied TOGA mRNA expression profiling to identify genes associated with the *in vitro* induction of M cell-like features in Caco-2 cells and tested them against normal Peyer's patch tissue for their expression in FAE. Among the genes identified by this method, laminin β3, a matrix metalloproteinase and a tetraspan family member, showed enriched expression in FAE of mouse Peyer's patches. Moreover, the *C. perfringens* enterotoxin receptor (CPE-R) appeared to be expressed more strongly by UEA-1$^+$ M cells relative to neighboring FAE. Expression of the tetraspan TM4SF3 gene and CPE-R was also confirmed in human Peyer's patch FAE. Our results suggest that while the Caco-2 differentiation model is associated with some functional features of M cells, the genes induced may instead reflect the acquisition of a more general FAE phenotype, sharing only select features with the M cell subset.

Introduction

The epithelium lining of the gut is for the most part impermeable to microorganisms and microparticles, except for the lymphoid follicle-associated epithelium (FAE) found in Peyer's patches (1–4). The FAE contains microfold (M) cells, which are scattered in the epithelium sheet covering lymphoid follicles of Peyer's patches, and which are responsible for transport of antigen, bacteria, viruses and microparticles to the antigen-presenting cells, within and under the epithelial barrier, as the first step in developing immune responses. There is only an incomplete and inadequate understanding of the development and function of FAE, and the genes and proteins responsible for their specialized functions remain to be identified.

One potential approach to studying such complex and specialized tissues is to use cell culture models that reproduce features of the *in vivo* tissue. Kerneis *et al.* (5) recently demonstrated that co-culturing human colon carcinoma Caco-2 cells with murine Peyer's patch lymphocytes appeared to convert the Caco-2 cells with M cell-like features including enhanced transport of particles across the epithelium monolayer. The induction of this phenotype did not require direct cell contact as it was also achieved in a transwell culture in which Caco-2 cells were cultured across a filter from RajiB cells (6). Although it is not clear that this model faithfully reproduces all of the features of M cell function *in vivo*, a

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comparison between genes induced in the Caco model and their expression in normal Peyer's patch tissue may provide insights to both the cell culture model and normal FAE and M cell biology.

In this study, we used gene expression profiling of the Caco-2 co-culture system to determine its potential for identifying genes associated with FAE and M cells in Peyer's patch tissue. The co-culture of Caco-2 cells with Raji B cells induces up- and down-regulation of numerous genes, including housekeeping genes, transcription factors and cell-surface proteins. Follow-on studies on expression of some of these genes in normal Peyer's patch tissue confirmed that the induction of Caco-2 cells *in vitro* allowed us to identify genes that were specifically expressed in Peyer's patch FAE *in vivo*. Interestingly, the genes identified were not uniquely expressed by M cells; however, they comprise a set of markers that may identify distinct subsets of FAE, and may provide important clues to FAE and M cell development and function.

Methods

Caco-2 cell culture

Caco-2 cells of passage 30–40 were maintained and grown on polycarbonate filters (3.0 μm) as described (6). The co-culture conditions were as follows: 5×10^5 RajiB cells were resuspended in RPMI:DMEM 1:2 and added to the basolateral chamber of 14-day-old Caco-2 cell monolayers and the co-cultures were maintained for 4–6 days. The corresponding mono-cultures of Caco-2 cells on matched filter supports were used as controls. The integrity of the cell monolayers was measured by transepithelial resistance before and after co-culture. Prior to RNA extraction, cells were washed once in medium before being scraped off the filters, pelleted and frozen in liquid nitrogen.

TOGA gene expression profiling

TOGA analysis was performed as previously described (7) on RNA samples isolated from cultured Caco-2 and RajiB cells. Briefly, RNA samples were converted to cDNA using a degenerate pool of biotinylated phasing primers that initiated synthesis at the beginning of the poly(A) tail on each mRNA. The cDNA collection was digested with the restriction endonuclease *Msp*I or *Sau*3A and the 3′ fragments were isolated by streptavidin-bead capture. The captured fragments were modified at their 5′ ends to harbor a start site for *in vitro* T3 transcription by ligation of an oligonucleotide adapter to the *Msp*I or *Sau*3A overhang. Following incubation of the modified fragments with T3 polymerase, a collection of RNA fragments was produced corresponding to the 3′ portion of each starting mRNA, from its most 3′ *Msp*I or *Sau*3A recognition sequence to the beginning of its poly(A) tail, with each fragment flanked by linker tags of known sequence. In the final step, 256 primers corresponding to all possible permutations of the four nucleotides immediately adjacent to the *Msp*I or *Sau*3A recognition site ($N_1N_2N_3N_4$) were matched with the appropriate N_1 template and utilized in individual robotically performed PCR paired with a fluorescent primer to produce 256 non-overlapping pools of products. The resulting products were separated by electrophoresis. This process assigned each product an address: an 8-nucleotide sequence (the four *Msp*I or *Sau*3A recognition and the adjacent four parsing nucleotides) and a length, both of which are attributes of the individual mRNAs. The amplitudes of the fluorescent PCR products correspond to the initial concentrations of their parent mRNAs and these were automatically collated into a database, indexed by the addresses, that could be queried electronically to identify mRNAs whose concentrations differed among the experimental samples. Peaks of interest were cloned from the TOGA libraries and sequenced. The gene identities were assigned based on database sequence matches from these sequences.

Real-time quantitative PCR (qPCR)

Quantitation of relative gene expression using qPCR was done with the ABI Prism 7700 system (Applied Biosystems, Foster City, CA). Reactions are characterized by the cycle number at which amplification of a PCR product is first detected. The higher the copy number of the nucleic acid target, the sooner a significant increase in SYBR I green fluorescence is observed. For each cDNA template, the cycle threshold (C_t) necessary to detect the amplified product was normalized to the C_t values of a housekeeping gene, human GAPDH. Relative differences in target abundance were calculated by the difference in cycle threshold at which an amplified product was detected ($\Delta\Delta C_t$ method). Reactions were performed in a 30-μl volume with 5 μM each forward and reverse primers (sequences listed in Table 3), 50 pg of cDNA (generated by DNase treatment of RNA, followed by SuperScript II synthesis), and SYBR Green PCR master mix with AmpliTaq Gold DNA polymerase. Gel analysis of reactions by ethidium bromide staining was done to confirm synthesis of a single amplicon of the correct size. Primer sequences were selected by the Primer Express software.

Tissue preparation, in situ hybridization (ISH) and immunohistochemistry

Fresh frozen sections of mouse, cynomolgus macaque and human Peyer's patches were sectioned 10 μm thick for both ISH and immunohistochemistry studies. All mouse studies were done using the BALB/cJ strain. Mouse and macaque tissue were dissected from normal animals sacrificed in accordance with NIH and institutional animal care and use guidelines. Human Peyer's patch tissue was acquired from surgical biopsies done for unrelated medical reasons; the blocks containing normal Peyer's patch tissue were confirmed by histological examination by a surgical pathologist. Sense and antisense riboprobes were synthesized and labeled with digoxygenin using SP6, T3 or T7 *in vitro* transcription. Hybridization signal was detected using an alkaline phosphatase-conjugated anti-digoxygenin antibody (Roche, Indianapolis, IN) with NBT/BCIP as a chromagen. Probes used for ISH listed by gene (TOGA address), GenBank accession nos and nucleotides: MTCAG137 (mouse X67280, nucleotides 72–1390), MTGGT175 (AF462391, nucleotides 418–1424), MGTTA124 (mouse AK008064, nucleotides 56–606), MTAAC201 (mouse NM_010700, nucleotides 1501–2545), SCTCG210 (mouse AI315052, nucleotides 92–482 and AI785618, nucleotides 1–551), SGGGA310 (mouse BC025461, nucleotides 12–711, human M35252, nucleotides

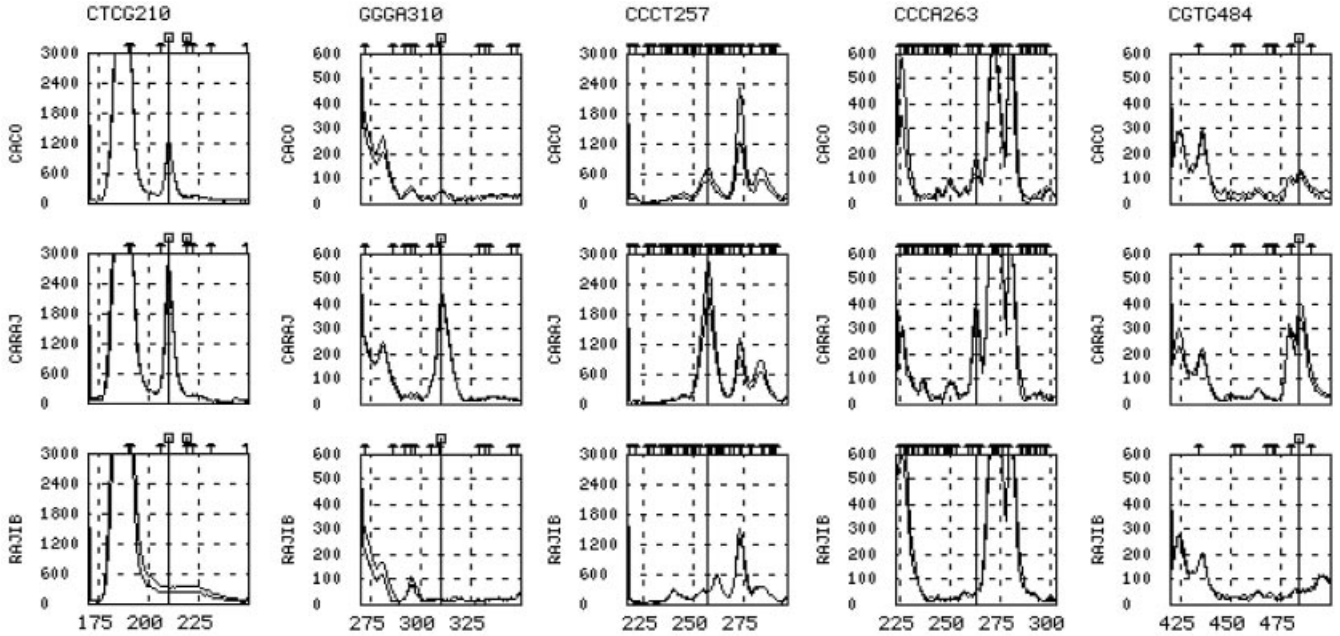

Fig. 1. TOGA profiles. Traces show peaks for indicated genes (identified by vertical lines) from Caco-2 cells (CACO), Caco-2/RajiB co-culture (CARAJ) and RajiB cells (RAJIB). Shown are trace data for the last five peaks in Table 2 (SCTCG210, SGGGA310, SCCCT257, SCCCA263 and SCGTG484).

182–858), SCCCT257 (mouse AB000713, nucleotides 171–806, human AB000712, nucleotides 183–816), SCCCA263 (mouse D86332, nucleotides 602–1962) and SCGTG484 (mouse U43298, nucleotides 2146–3646). Human probe sequences were used for cynomolgus macaque tissue. For co-localization studies, ISH signals were detected using peroxidase-conjugated anti-digoxygenin (Roche) followed by TSA–FITC amplification (TSA-Plus; NEN, Boston, MA), followed by staining with anti-Claudin-4 antibody (Zymed, San Francisco, CA), followed by biotinylated rat anti-mouse IgG1 and avidin–FITC or rhodamine-conjugated UEA-1 (Vector, Burlingame, CA). For the Claudin-4 staining, control staining with no primary antibody only showed background staining of mouse plasma cells within the Peyer's patch follicles and no FAE signal.

Results

Gene expression profiling of Caco-2 co-cultures

The original goal of our study was to find cell-surface molecules that may explain the apical-to-basolateral transcytosis function in M cells, since such molecules might also be used as target receptors for delivery of synthetic vaccines. We used the cell culture model described first by Kerneis *et al.* (5) and modified by Gullberg *et al.* (6). Here, Caco-2 monolayers were grown on a membrane filter across from Raji B cells, so that RNA isolated from the Caco-2 cells was not contaminated by Raji B cells. Studies by Gullberg *et al.* (6) have shown that Caco-2 differentiation is induced despite the cell separation, implying that the inducing factors are soluble cytokines produced by the B cell line. We only used cultures shown to retain high transepithelial resistance and possess the ability to transcytose microparticles. For technical reasons, the percentage of induced cells in each culture could not be determined prior to harvesting.

To identify genes associated with the acquisition of M cell and FAE function we compared mRNAs expressed in Caco-2 cells before and after RajiB co-culture using TOGA (7), an automated open system PCR-based mRNA-profiling method that requires only small amounts of tissue. We isolated RNA samples for TOGA analysis from cells generated in the modified version of the Caco-2 co-culture system (6): (i) Caco-2 cells cultured alone ('Caco'), (ii) Caco-2 cells cultured with RajiB cells, but separated across the transwell barrier ('CaRaj'), and (iii) RajiB cells cultured alone ('RajiB'). Expressed sequences are identified in TOGA data sets as peaks on a trace from each of 256 different PCR reactions, with peak amplitude being roughly proportional to the level of gene expression (e.g. see Fig. 1). TOGA robotically indexes these RNAs by an 8-nucleotide identifier sequence and its distance from the poly(A) tail, and compiles the relative amount of each mRNA in an electronic database. Analysis of the TOGA data detected expression of ~15,000 mRNAs using the restriction enzyme *Msp*I and 11,500 mRNAs using *Sau*3A.

Since we were primarily interested in possible receptor genes that would be significantly up-regulated by co-culture, and low or absent in uninduced Caco-2 cells and Raji cells, the data sets were queried for those mRNAs with ⩾2-fold relative expression in the co-culture sample than either the uninduced Caco-2 or the RajiB cells. This analysis yielded 89 mRNAs in the *Msp*I data and 149 mRNAs in the *Sau*3A data, together comprising <1% of total identified mRNAs. Identification of the expressed genes depends on direct cloning of the peaks, so cDNA clones for most of these differentially expressed mRNAs were isolated and their nucleotide sequences were used to

Table 1. Selected genes identified by TOGA gene expression profiling: genes encoding transcription factors and other genes

Gene (accession no.)	Caco	CaRaj	RajiB	Fold regulation by TOGA, qPCR
Up-regulated				
transcription factor (AK000232)	44	2165	51	49.2, 119.3
jagged-1 (AW369026)	43	328	46	7.6, 1.8
c-Maf (AV648578)	134	1273	49	9.5, 14.7
DEC-1 (AB004066)	169	972	78	5.8, 3.7
RAB-13 (W46375)	73	345	23	4.7, 9.3
glutaredoxin (AW128930)	135	890	270	6.6, 3.6
GPx-4 (X71973)	92	277	55	3.0, 4.4
ULK1 (AF045458)	119	333	61	2.8, 2.5
CDC2-related kinase (Q14004)	56	149	53	2.7, NT[a]
Down-regulated				
ubiquitin B (BC000379)	1492	665	979	0.45[b]
mitochondrial gene (E27671)	3360	138	3918	0.04[b]
3-pgdh (AF006043)	1091	268	997	0.25[b]
farnesyl diphos synthase (BC010004)	2066	831	2168	0.40[b]
transketolase (BC008615)	1123	416	1155	0.37[b]

Numbers reflect peak amplitudes in the TOGA data set for the gene listed and is an average from two independent TOGA libraries. Fold-regulation was calculated as the ratio of peak amplitudes of CaRaj to Caco and by qPCR (primers listed in Methods). CaRaj; Caco-2/Raji co-culture sample.
[a]Not tested.
[b]qPCR was not performed for these genes.

Table 2. Selected genes identified by TOGA gene expression profiling: candidate receptor genes up-regulated in Caco-2 co-cultures

Gene	Address	Fold-regulation by TOGA, qPCR	Expression
Biliary glycoprotein A	MTCAG137	3.9, 1.8	FAE = villi
μ Protocadherin	MTGGT175	10.6, 19.5	FAE = villi
Tetraspan TM4SF5	MGTTA124	2.6, 1.0	FAE ⩾ villi
LDL-R	MTTAC201	5.0, 1.1	FAE > villi
Apolipoprotein B	SCTCG210	2.3, 3.5	FAE > villi
Tetraspan TM4SF3	SGGGA310	8.9, 4.8	FAE >> villi, crypts
CPE-R	SCCCT257	4.2, 1.8	FAE, M cells, villi
MMP15	SCCCA263	2.7, 0.7	FAE
Laminin β3	SCGTG484	3.0, 1.9	FAE

The TOGA address refers to the enzyme used to generate the cDNA tags (M for *Msp*I, S for *Sau*3A), the 4-base parsing sequence and the length of the tag generated. Thus, MTCAG137 is a tag generated using *Msp*1, the TCAG parsing primer in the amplification and the resulting tag was 137 nucleotides long. The fold induction was calculated as the ratio of the peak amplitudes in the Caco-2 co-culture sample to the Caco-2 sample or using qPCR (primers listed in Methods). Expression of the gene in mouse tissue is based on ISH data using digoxygenin-labeled riboprobes specific for the gene listed (with accession nos for mouse and human homologues listed in Methods), and is based on visual observations from multiple experiments with several (more than three) animals and at least two different readers.

search nucleic acid databases. Sequence matches were used to assign gene identities.

Among the genes identified in this group of up-regulated mRNAs, many encoded 'housekeeping' genes, signaling molecules, cytoplasmic proteins and transcription factors. A selection of these genes with significant up-regulation is listed in Table 1. Confirmation of the up-regulation of some of these genes was also provided by qPCR (Table 1). It is not known whether these genes are specifically involved in the M cell-like phenotype of the induced Caco-2 cells; indeed, many of the genes may be regulated only in response to changes in cell cycle regulation as the Caco-2 cells differentiate. For example, among genes showing significant *down*-regulation in the co-cultures relative to Caco-2 and Raji cells (Table 1), many of these genes appeared to be 'housekeeping' genes that may simply reflect changes in the metabolic state of the differentiating Caco-2 colon carcinoma cells.

In view of these results, the total set of up-regulated genes identified was only considered a list of 'candidate' differentiation-associated genes. That is, regardless of the predicted fold-regulation of the candidate genes, we also required confirmation of expression within the target cell type *in vivo*. Moreover, to narrow our focus to candidate genes as possible M cell receptors, we selected the subset of genes encoding predicted cell-surface proteins based on GenBank assigned identities and functions, and proceeded to assess their expression in normal Peyer's patch tissue. A partial list of these genes is presented in Table 2 showing induction estimated by both TOGA and qPCR; the TOGA peak traces for selected genes are shown in Fig. 1.

Table 3. Primer sequences used in qPCR studies

Gene	Forward primer	Reverse primer
Biliary glycoprotein A	GCAGCTGTCCAATGGCAAC	GTCATTCCTTGTGACACTGAGTAGAGT
μ Protocadherin	CCCAAGGCTTTGACAACCAG	CCCAGTTGGCCTTGTGGTC
Tetraspan TM4SF5	ACACACCTCACTGAGGCTCCA	TCCAGGAAGGAGCAGGTGTAA
LDL-R	CCCATCCTCCCGACCC	GAAATGCAAGGAGACCACGG
Apolipoprotein B	GCCAAACTGCTTCTCCAAATG	TCTTGGAAACTGTGGAGCCAT
Tetraspan TM4SF3	GATCGGGAACAAATGAATCTGTG	GGTTTGACTGACGATAGCTTGATG
CPE-R	CCCTCTGAGTCCTCTGCCC	CACCCTCCCAGGCTCATTAGT
MMP15	ATCCCCAAGGCACTTGAGG	AACCACTCGCAGAGGCCA
Laminin β3	GCCAATGGGACAGTTACGCT	GCATGCCAATCTCCACCATC
Transcription factor	CCTTTGGGAAATCACATGCC	GCCATCTCGGTTGTGGCT
Jagged-1	GGCACGCCGACAAAACA	AGCTCTGGGCACTTTCCAAGT
c-Maf	TTGCACAATGTTCAATGATCTCAG	GCTTCAAAGGTGATCAACGTTTC
DEC-1	CAGAAAATCATTGCCCTGCA	GAATCAGCACTCACCAGCTTGT
RAB-13	GCACATCCCTTCTGCTTCCTC	GATGGAAGGAGAGTGGGCTTC
Glutaredoxin	GTGCAGAGGCTGTGGTCATG	GGATAGCCATCTTAAATAACAGAGTGTTC
GPx-4	GGGAAATGCCATCAAGTGGA	GCCGTTCTTGTCGATGAGGA
ULK1	CTGCCCCTGGTTGAATGTT	AAAATAGACAAAGGGTCCAGCACT

Peyer's patch FAE expression of candidate receptor genes

Among the candidate receptor genes induced in Caco-2 cells, confirmation of their association with genuine M cells *in vivo* depends on identification of their transcripts in Peyer's patch FAE. Thus, we cloned mouse homologues for the mRNAs originally identified in the human Caco-2 cultures and used them as probes for ISH studies on frozen sections of mouse Peyer's patches (summarized in Table 2). In some cases, ISH revealed expression specific to all intestinal epithelium, whether associated with Peyer's patches or not (Fig. 2a). The results with these genes are consistent with the possibility that regulation of many of the epithelium-specific genes in the Caco-2 co-cultures reflects only some general changes in the metabolism or cell cycle of differentiating carcinoma cells.

Several of the mRNAs showed expression specific to Peyer's patch FAE. These genes showed expression limited to the epithelium overlying the mouse Peyer's patch tissue, with little or no expression in villi (Fig. 2b). These included the genes for the laminin β3 chain, the membrane-type 2 matrix metalloproteinase (MMP15) and the tetraspanin TM4SF3. As shown in Fig. 2(b), expression of laminin β3 and MMP15 was relatively uniform across the face of the Peyer's patch, although occasional FAE cells appeared to be negative for expression. Expression of the tetraspanin TM4SF3 gene was less uniform across the FAE; moreover, its expression was also detected in crypt cells throughout the small intestine.

We next examined the co-localization of expression of these genes with the binding of the mouse M cell-specific lectin, UEA-1. This lectin is specific for exposed fucose moieties, restricted to M cells in the mouse Peyer's patch (8,9). While UEA-1 staining shows a circular pattern in epithelial M cells, it is not specific for the enclosed lymphocytes, as lymphocytes in the neighboring lymphoid follicle remain negative for UEA-1 binding. Most of the mRNAs tested were expressed by both UEA-1$^+$ and UEA-1$^-$ cells within the FAE. However, in the case of the laminin β3 and apolipoprotein B (not shown), their expression appeared to be predominantly in UEA-1$^-$ cells, with little or no co-localization with UEA-1$^+$ cells. The expression of the *C. perfringens* enterotoxin receptor (CPE-R) showed a more interesting pattern; although expression was also detected at the tips of intestinal villi, within the FAE the highest expression was primarily co-localized with UEA-1$^+$ cells, evident both by ISH and antibody staining (Fig. 3). In addition, while the protein appeared to be localized to epithelial tight junctions in villus epithelium and FAE, its distribution in M cells appeared to be cytoplasmic (see also below). This staining does not appear to be specific for embedded lymphocytes, as nearby lymphoid follicles are negative for staining.

The mRNAs we found to be FAE enriched in mouse were originally identified from the human Caco-2 cell line, so we tested whether the human homologues of these genes were also expressed specifically in human FAE. The human TM4SF3 and CPE-R were expressed in a subset of FAE cells from two human donors (Fig. 4), although the human homologues for laminin β3 and MMP15 were not detectable above background using non-radioactive riboprobes. Here too, CPE-R protein appeared to be predominantly cytoplasmic in the strongly positive cells of the FAE, although fine staining in an epithelial tight junction pattern could also be detected. FAE-specific expression of CPE-R was also detected in cynomolgus macaque Peyer's patch tissue (Fig. 4b). Thus, at least some of the mouse FAE-specific genes were also found in human and macaque FAE, though we have not yet confirmed specific expression among human or macaque M cells. It has been reported that an antibody against sialyl-Lewis A antigen identifies human M cells (10), but we have not achieved specific staining with this reagent in our hands, although technical differences may account for our results.

Discussion

Does the Caco-2 model reproduce M cell differentiation?

It might not be fair to ask whether the Caco-2 induction reproduces the M cell phenotype, since there are few known molecular correlates of the M cell phenotype. Thus, to help generate a better molecular profile of M cells, we used a gene expression profiling strategy to identify several mRNAs associated with FAE, including genes expressed by (although not

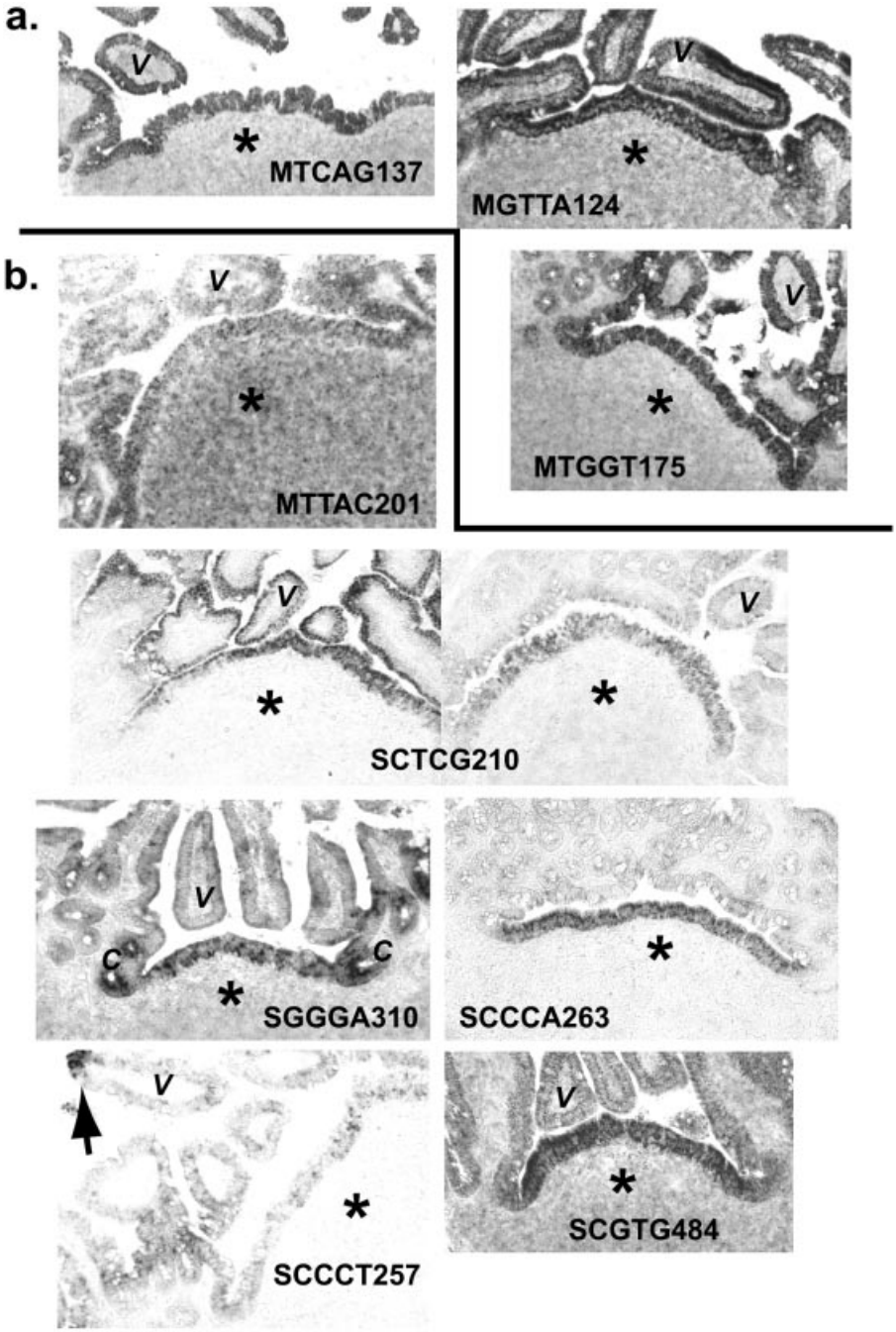

Fig. 2. ISH of mouse Peyer's patch. (a) Genes showing epithelial expression; (b) genes showing higher expression in FAE relative to villi. For clarity, the images are identified by their TOGA addresses (MTCAG137, biliary glycoprotein A; MGTTA124, tetraspan TM4SF5; MTGGT175, μ protocadherin; MTTAC201, LDL-R; SCTCG210, apolipoprotein B; SGGGA310, tetraspan TM4SF3; SCCCA263, MMP15; SCCCT257, CPE-R; SCGTG484, laminin β3); villi are indicated by 'V', the Peyer's patch follicle is indicated by an asterisk and crypts are marked by (C). The expression of SCTCG210 was variably detected on villi and FAE, so two representative images are shown. In the case SCCCT257, hybridization signal was detected on both scattered cells in the FAE and at the tips of some villi (V), shown by an arrow. Original magnification of the photographs was ×200.

exclusive to) M cells. The use of the Caco-2 cell culture model led to the identification of one gene (CPE-R) with enriched expression in mouse M cells, with similar distribution in human Peyer's patch FAE. Final confirmation of M cell expression in human FAE will require additional study, including detailed ultrastructural studies on cells both in human Peyer's patch and Caco-2 cultures. Other genes identified from the Caco-2 model showed less consistent expression patterns in mouse and human FAE; these differences may reflect species differences in Peyer's patch development and function or they may reflect regulation of genes not related to the acquisition of M cell-like function.

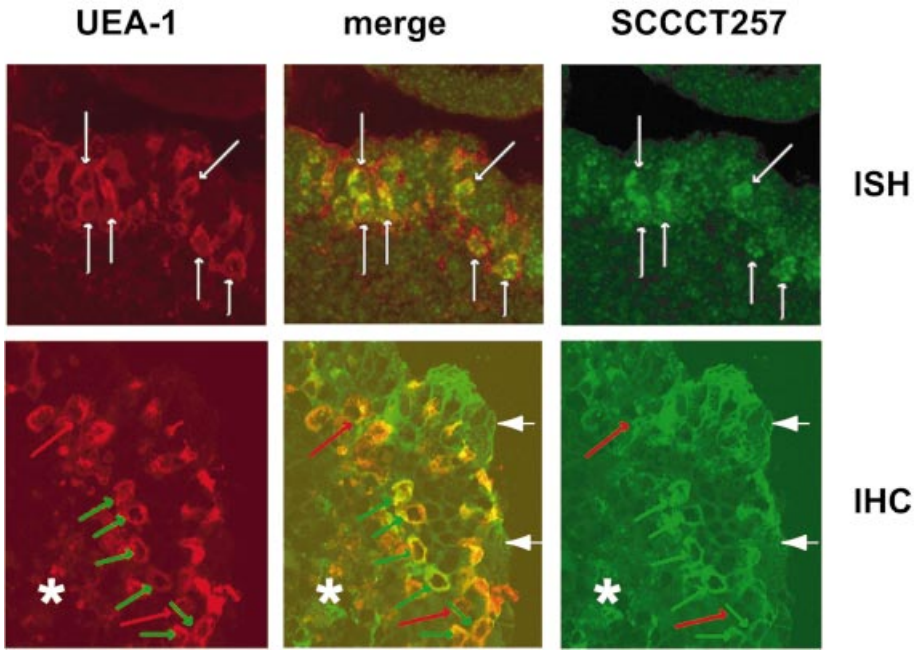

Fig. 3. Co-localization gene/protein expression with UEA-1. FAE-specific gene expression (green) by ISH and immunohistochemistry for CPE-R (SCCCT257) was compared to UEA-1 lectin binding (red) in mouse Peyer's patches. Co-localization of signal was present in most UEA-1$^+$ cells (green arrows), although expression was also detected on subsets of villus epithelium and weak linear staining for protein was detected on most enterocytes in an intercellular tight junction pattern (arrowheads). Circular staining of M cells by UEA-1$^+$ and antibody to CPE-R was not cross-reactive for lymphocytes, as neighboring lymphoid follicle is negative for staining (asterisk). Original magnification ×630.

Previously, it has been a challenge to identify proteins positively associated with increased or specific expression in FAE in any mammalian species (1–4,9,11), although one recent paper suggested that the expression of the lymphocyte chemokine receptor CCR5 may be specifically expressed in human FAE (12) and a novel as yet unidentified IgA-binding activity has been localized to M cells (13). In contrast, expression of enterocyte-specific proteins such as brush border hydrolases and alkaline phosphatase was reduced in FAE and M cells (14,15). We did not detect regulation of these genes in our TOGA analysis, in part because genes such as CCR5 do not have *Msp*I or *Sau*3A restriction sites near the 3′ end of the expressed mRNA transcript, so they would not be present in the TOGA data sets. However, the open system gene expression profiling did identify regulated genes that might not have been studied by other more focused approaches. Our strategy, especially the analysis of tissue expression by ISH, also allowed identification of diverse patterns of regulation among FAE subsets.

Thus, it was curious that for some of the genes induced in Caco-2 cells, such as MMP15 and laminin β3, expression of the mouse homologue was enriched in mouse FAE, but not necessarily with UEA-1$^+$ M cells. Indeed, while the Caco-2-induced genes were originally presumed to be associated with differentiation toward an M cell-like phenotype, some of the genes may turn out to be restricted only to non-M cells of the mouse FAE. For example, laminin β3 expression may be nearly exclusive to the non-M cell FAE, although a recent study suggested that general laminin expression in the FAE was uniform and similar to that in the villi (16). Laminin β3 was not specifically examined in that study, however. The laminin β3 chain encodes a protein associated with the basement membrane, hence the transcytosis function of M cells might favor the absence of basement membrane connections.

Together, these data suggest that Caco-2 differentiation *in vitro* does not exclusively reproduce all features of the M cell phenotype. That is, co-culture may instead involve the induction of genes associated with several related FAE phenotypic subsets, including, but not limited to, the M cell subset. This conclusion of course also presumes that the FAE includes several related overlapping phenotypes, with M cells representing only one end of the spectrum of FAE cell types. This is consistent with earlier reports in which antibody and ultrastructural studies indicated heterogeneity in FAE and M cell-enriched preparations (17–19); these genes may now provide molecular markers to distinguish among these subsets. It is important to note that this spectrum of gene expression phenotypes was evident in the analysis of gene expression in both normal mouse and human Peyer's patch tissue, and would not be affected by any potential heterogeneity among Caco-2 cells. Additional studies on the Caco-2 co-culture may reveal whether the induced cells show a differentiation phenotype that reflects mixed or intermediate characteristics of various FAE subsets, or alternatively whether there is considerable heterogeneity among the induced cells.

What do the FAE-specific genes tell us about FAE and M cell biology?

Our results do provide some interesting insights into FAE and M cell biology. While the transcytosis function of M cells helps

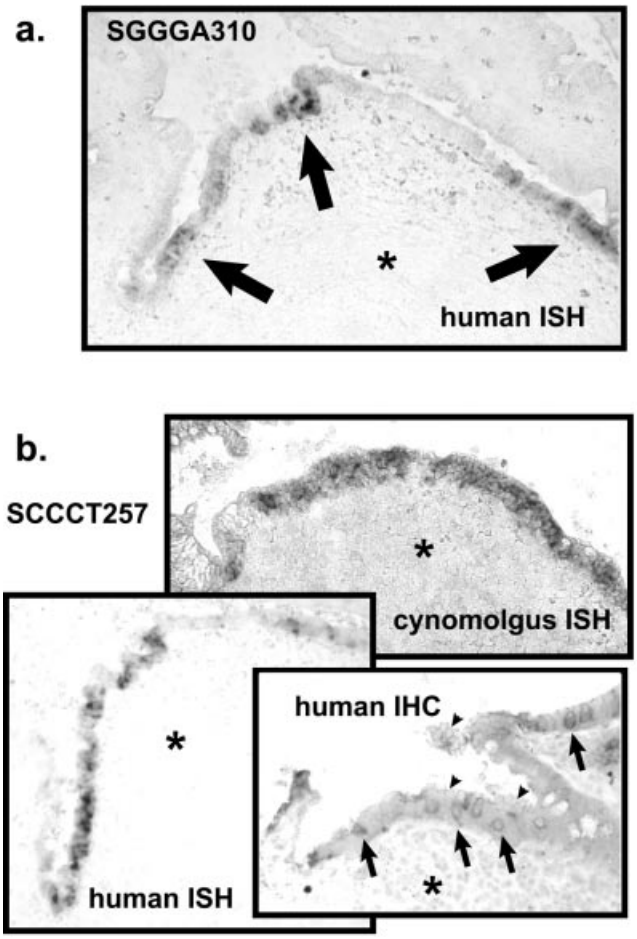

Fig. 4. Expression of TM4SF3 (SGGGA310) and CPE-R (SCCCT257) in human and cynomolgus macaque Peyer's patch. (a) For SGGGA310, the arrows identify patches of detectable ISH signal in the human FAE. (b) For SCCCT257, ISH signal is detectable in both cynomolgus macaque and human FAE. The human immunohistochemistry panel shows staining of human FAE using an antibody against CPE-R, with hematoxylin counterstain; arrows indicate cells with strong cytoplasmic staining and arrowheads indicate fine linear staining of epithelial tight junctions. The Peyer's patch follicle is indicated by an asterisk. Original magnification ×200.

distinguish them from conventional enterocytes, less is known about features distinguishing the general FAE cell phenotype from that of other enterocytes. Reports of characteristic glycosylation patterns in the FAE (9) and the FAE expression pattern found for a hybrid transgene (11) suggest that FAE has its own specific program of gene regulation. The studies presented here help define the FAE in molecular detail by identifying for the first time some of the particular proteins associated with FAE and M cells in mammalian Peyer's patch.

Some of these proteins have predictably important roles in epithelial development. For example, MMP15 belongs to the family of membrane-type matrix metalloproteinases; family members are known to be involved in tissue inflammation, wound healing, tumor metastasis and regulation of epithelial integrity (20,21). TM4SF3 and TM4SF5 belong to the tetraspan family, whose members have been reported to be important in several signaling pathways and epithelial differentiation (22,23), e.g. the tetraspan protein CD63 was recently shown to be important in differentiation of an intestinal epithelial cell line (24). Finally, CPE-R is also known as claudin-4, a member of a family of proteins associated with epithelial tight junctions (25,26). Its role in M cell function is not known, but its dual function as a tight junction protein and a receptor for a bacterial enterotoxin may indicate an important role in M cell transcytosis of pathogens. Indeed, the apparent differences in protein localization in enterocytes (tight junctions) versus M cells (cytoplasmic) suggest a unique function in M cells.

While our results suggest the co-existence of several FAE phenotypic subsets, including M cells, it is not clear how their development is differentially induced and whether there is a 'master switch' which controls such differential induction. The general FAE phenotype appears to be dependent on the lymphoid cells residing in the underlying Peyer's patch (27) and cytokines such as lymphotoxin β (28,29) may be the main inductive agents in this system. A general set of factors directed at all epithelial cells overlying the Peyer's patch may give rise to a general FAE phenotype, including expression of the genes described in the present report. Additional factors, directed or stochastic, may then provide triggers for the development of M cell function. However, the specific triggers of M cell development [e.g. direct lymphocyte contact (5,6,11,27)] and the stage at which M cell differentiation is initiated [i.e. from crypt cell precursors or from mature FAE (30,31)] remain to be determined. Moreover, the patterns of gene expression identified so far do not yet clearly suggest any FAE subset lineage relationships (e.g. M cell precursors versus non-M cell precursors). Study of the promoter sequences of FAE and M cell-specific genes should provide important clues to these triggering events.

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Abbreviations

CPE-R	*C. perfringens* enterotoxin receptor
FAE	follicle-associated epithelium
ISH	*in situ* hybridization
M cell	microfold cell
qPCR	real-time quantitative PCR

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