Effects of lifestyle and environmental factors on intestinal epithelial cell morphogenesis
Klunder, Leo Jan

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Klunder, L. J. (2016). Effects of lifestyle and environmental factors on intestinal epithelial cell morphogenesis. [Thesis fully internal (DIV), University of Groningen]. Rijksuniversiteit Groningen.

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 5

Cigarette smoke impairs intestinal epithelial cell polarity development and glandular morphogenesis

Leon J. Klunder¹, Tjasso Blokzijl², Rosaria Ferreira¹, Klaas Nico Faber², Gerard Dijkstra²*, Sven C. D. van IJzendoorn¹*

¹Department of Cell Biology, ²Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands
*These authors contributed equally

Manuscript in preparation
Abstract

Cigarette smoking is emerging as an important lifestyle factor that affects intestinal function. Epidemiological studies indicate that cigarette smoking influences the course of inflammatory bowel disorders and is a risk factor for colorectal cancer and hyperplastic intestinal polyp development. The mechanism by which cigarette smoke exerts its impact on the intestinal epithelium is unclear. These may involve indirect mechanisms via alterations in the immune system and/or direct actions of cigarette smoke on the intestinal epithelium. Little is known about the direct effects of cigarette smoke on intestinal epithelial cell behavior. In this study, we employed a 3D human intestinal epithelial Caco-2 cell spheroid model to investigate the effects of cigarette smoke extract (CSE) on intestinal epithelial barrier function, cell polarity and morphogenesis. We found that CSE did not affect the polarity and barrier function of pre-established lumen-forming Caco-2 spheroids. However, when exposed to CSE during the process of three-dimensional morphogenesis, cells showed dose-dependent impairment of growth and de novo development of apical-basal cell polarity and tight junctions, resulting in defective self-assembly into single lumen-forming spheroids. These effects penetrated into future cell generations. The administration of the glutathione precursor N-acetyl-L-cysteine (NAC) completely neutralized the inhibitory effects of CSE in a manner that involved glutathione-dependent and –independent mechanisms. Our data demonstrate that CSE has direct inhibitory effects on intestinal epithelial cell polarity development and morphogenesis, and suggests a potential clinical application of NAC in the treatment of smoking-related intestinal disorders.
Introduction

The intestinal epithelium and its homeostasis is subject to influences from many life-style and environmental factors. Epidemiological studies indicate that cigarette smoking has a strong influence on the intestinal epithelium. Cigarette smoking influences the course of Crohn’s disease and ulcerative colitis, two predominant inflammatory bowel disorders that, at the epithelial level, are characterized by impaired intestinal barrier function. Impaired intestinal barrier function is also associated with the smoking-related lung disease chronic obstructive pulmonary disease (COPD). Furthermore, cigarette smoking is a risk factor for hyperplastic intestinal polyp development, as well as for colorectal cancer, which is one of the leading causes of death in the Western world. Animal studies support the correlation between cigarette smoking and its negative effects on the intestinal epithelium. Exposure of normal laboratory mice to cigarette smoke led to impaired intestinal barrier disruption, increased apoptosis and autophagy in the ileum, decreased cystic fibrosis transmembrane conductance regulator (CFTR) activity in the intestinal epithelium, increased bacterial translocation, intestinal villi atrophy and small/large intestine-specific alterations in tight junction protein expression. Furthermore, cigarette smoke exposure has been reported to aggravate intestinal inflammation in trinitrobenzenesulfonic acid-induced colitis in rats. The mechanism by which cigarette smoke exerts its impact on the intestinal epithelium is unclear. Many cell types contribute to intestinal epithelial homeostasis, including intestinal epithelial cells that are constantly renewed through intestinal stem cell activity, complex communities of symbiotic microorganisms in the gut lumen (the microbiota), and various immune cell populations in the lamina
Effects of interferon-gamma and energy metabolism pathways on intestinal epithelial gland morphogenesis in three-dimensional culture

propria. Not surprisingly therefore, the mechanisms by which cigarette smoking influences intestinal epithelial homeostasis have been proposed to involve alterations of the immune system\(^\text{18}\) and/or the intestinal microbiota\(^\text{15,19–22}\). Little is known, however, about the potential direct effects of cigarette smoke on intestinal epithelial cell behavior\(^\text{23}\). In order to investigate potential direct effects of cigarette smoke on intestinal epithelial cell behavior, a reductionist in vitro approach is required in which other cell types that are normally present in the intestine (e.g., bacteria, immune cells) are excluded. Several in vitro model systems to study intestinal epithelial cell biology are available. Of these, two-dimensional (2D) cell culture, in which cells are grown on flat polystyrene or glass substrata, is most commonly used. However, these substrata are unnatural and do not allow cells to organize themselves in a three-dimensional environment as they do in vivo. Moreover, 2D cell cultures are poor predictors of cellular toxicity and toxigenic responses\(^\text{24–27}\). To overcome these and other drawbacks of 2D cell culture, three-dimensional (3D) cell culture systems have been developed. In 3D cultures single epithelial cells are mixed with extracellular matrix/basement membrane components, which allows the proliferating epithelial cells to establish natural cell-matrix and cell-cell attachments and to self-organize to form a multicellular polarized architecture that best reflects the physiological in vivo situation\(^\text{28–32}\). 3D cell culture systems therefore provide a suitable approach bridging the gap between 2D cell culture and in vivo animal models/humans\(^\text{33}\).

In this study, we employed a recently developed 3D in vitro intestinal epithelial cell culture system\(^\text{34–38}\) to investigate the effects of cigarette smoke extract on intestinal epithelial cell polarity, barrier function and morphogenesis.
Materials and Methods

**Three-dimensional Caco-2 cell culture.** Three-dimensional (3D) culture of human Caucasian colon carcinoma-2 (Caco-2) cells (ATCC) was performed as described previously. Briefly, Caco-2 cells were grown in culture medium (Dulbecco’s modified Eagle’s medium (DMEM) supplemented with glutamine, 10% fetal bovine serum (FBS) and antibiotics). Cells were grown to ~85% confluence in a 25 cm² tissue culture flask, detached with trypsin and ethylene diamine-tetraacetic acid (EDTA) dissolved in phosphate-buffered saline solution (PBS), re-suspended in culture medium supplemented with 2% (v/v) Matrigel, and plated at a concentration of $1.5 \times 10^4$ cells/ml in wells of a 8-well plate pre-coated with 100% (v/v) Matrigel for up to 7 or 8 days. With this protocol, Caco-2 cells developed spheroids consisting of a monolayer of cells with functional apical-basal cell polarity and tight junctions that surround a central apical lumen.

**In vitro exposure of cells to CSE.** Cigarette smoke extract (CSE) was prepared as described by passing smoke from 2 standard 3R4F research cigarettes (Tobacco research institute, Kentucky 3R4F, University of Kentucky, KY, USA) through 25 ml culture medium, which was defined as 100% CSE. Freshly-prepared CSE was used within 30 min and diluted directly into culture medium for the indicated concentrations. Cells were exposed to CSE, N-acetyl-L-cysteine (NAC; 1 mM), L-buthione-S,R-sulfoximine (BSO; 10 uM) from the day of plating for various time intervals. In some experiments living cells were subsequently used to determine epithelial barrier integrity (see below). In other experiments the culture medium was removed and the cells were fixed with 4% paraformaldehyde (PFA) and processed.
for immunofluorescence labeling (see below). CSE-treated cells under conditions tested did not show changes in cell viability that were statistically significantly different from controls.

**Immunofluorescent labeling, microscopy and digital image processing.** 3D cell cultures were fixed with 4% paraformaldehyde at 37°C for 1 h, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, Selze/Germany) at room temperature for 20 minutes and washed with Hank’s balanced salt solution (HBSS). Following blocking in 3% bovine serum albumin (BSA) in HBSS at 37°C, spheres were immunolabeled with polyclonal rabbit antibodies against β-catenin (BD Biosciences), polyclonal rabbit antibodies against ZO-1 (1:100; Invitrogen, Carlsbad/USA) or monoclonal mouse antibodies against ZO-1. All primary antibody incubations were performed at 37°C for 1.5 h. Samples were subsequently washed with HBSS and labeled with secondary Alexa-Fluor488 conjugated goat anti-rabbit or Alexa-Fluor488 goat anti-mouse antibodies (1:1,500; Molecular Probes, Leiden/The Netherlands). Filamentous actin was visualized with tetramethylrhodamine isothiocyanate (TRITC)-phalloidin (1:1,000 Sigma-Aldrich) or TRITC-phalloidin-688 (1:1,000 Sigma-Aldrich). Nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI; 1:1,000). Cells were examined with a Leica DMI6000B inverted fluorescence microscope or a Leica SP8 confocal laser scanning microscope (Leica Microsystems). Images were processed using LAS AF Lite (Leica Microsystems) and ImageJ software.

**Measurement of tight junction integrity.** Tight junction integrity of 3D Caco-2 monolayers was performed as described previously. Briefly, control and CSE-treated Caco-2 spheroids were exposed to fluorescein isothiocyanate (FITC)-dextran of 4 kDa (FD4; Sigma-Aldrich, Selze/Germany)
at 37 °C for 1 h. Cells were then examined with a fluorescence microscope to determine whether FD4 has leaked into the spheroid lumens. In case of impermeable monolayers, no FD4 leakage into the spheroid lumen is observed \(^{36-38}\).

**Measurement of glutathione levels.** Cells were seeded in a 6-well plate cultured for 3 days to form a monolayer. Cells were incubated with CSE in the presence or absence of NAC and/or BSO for 24 h. Glutathione levels were determined spectrophotometrically assessing the reaction with glutathione reductase, using the Hitachi u-29000 spectrophotometer at 30°C for 10 minutes, measuring every 60 seconds.

**Statistical analyses.** The percentage of lumen-forming cell clusters under different treatment conditions were compared using \(\chi\)-square tests, sphere sizes were compared using t-tests.

**Results**

**Cigarette smoke extract does not perturb the integrity of preformed Caco-2 cell spheroids**

Caco-2 cells in three-dimensional culture, consisting of the basement membrane extract (Matrigel), self-assembled to form spheroids over a period of 7 days that comprised a monolayer of cells that surrounded a single central (apical) lumen. The cells in these monolayers were highly polarized, with a dense actin network at the luminal side (reflecting the presence of actin-based microvilli \(^{36,38}\)), and tight junction proteins at the apex of the lateral plasma membrane between neighboring cells \(^{36,38}\) (Figure 1A). The functionality of these tight junctions was confirmed by the exclusion of basolaterally-administered FITC-labeled 4 kDa dextran (FD4) from the apical lumen (Figure 1C). Treatment of the spheroids with ethylene glycol tetraacetic acid (EGTA), a calcium
Figure 1. Cigarette smoke extract does not perturb the integrity of preformed Caco-2 cell spheroids. A) Caco-2 cells developed single lumen-forming spheroids during 7 days in 3D culture, as evidenced by the monolayer organization of cells (nuclei is visualized by DAPI in blue) that display an apical enrichment of actin (labeled with TRITC-phalloidin in red) and ZO-1 (labeled with anti-ZO-1 antibodies in green). B) Spheres pre-formed as in A, treated with 10% CSE for 24 h. Staining as in A.C) Exposure of Caco-2 spheroids (shown by white light microscopy images) to CSE did not lead to the leakage of FITC-labeled dextran (FD4, green) into the spheroid lumen, whereas treatment with the calcium chelator EDTA did. D) Quantification of the results depicted in 1B (see Materials and methods). Data in panel C are presented as mean ± SEM of at least three independent experiments. Boxes in panels A and B indicate the area of which the enlarged images are shown. Asterisks indicates the location of the apical lumen. Bars: 30 um.
Effects of interferon-gamma and energy metabolism pathways on intestinal epithelial gland morphogenesis in three-dimensional culture

A chelator that perturbs tight junction integrity led to the leakage of FD4 into the apical lumen, as qualitatively shown by fluorescence microscopy of living cells (Figure 1C), and quantitatively by an increase in the ratio of FD4 fluorescence intensity [basal]/FD fluorescence intensity [lumen] (Figure 1D), and was indicative of increased paracellular permeability. Treatment of the spheroid cultures with increasing concentrations of CSE for up to 24 hours did not lead to alterations in the general appearance of the spheroids, did not lead to the leakage of FD4 into the apical lumen (Figure 1C, D) and did not alter the subcellular distribution of the tight junction-associated protein ZO-1 (Figure 1B), indicating that CSE did not perturb the tight junction function and barrier integrity of pre-formed Caco-2 spheroids.

**Figure 2. Cigarette smoke extract inhibits Caco-2 cell morphogenesis in 3D culture.** White light microscopical images of Caco-2 cell clusters formed during 7 days of 3D culture in normal culture medium or normal culture medium supplemented with CSE. Note the presence of a monolayers of cells surrounding a single central lumen in control cells and the absence of a monolayer organization and lumen in CSE-exposed clusters. Asterisk indicates the location of the apical lumen. Bars: 30 um.

**Cigarette smoke extract inhibits morphogenesis in Caco-2 3D cultures**
Under standard 3D culture conditions Caco-2 cells...
formed multicellular, single lumen-forming spheroids as a function of time (c.f., Figure 1A). Thus, 7 days after plating cell clusters displayed a typical donut-shaped morphology, as readily visualized by light microscopy (Figure 2) \(^{34-36}\). In contrast, when the culture medium of Caco-2 cells was supplemented with CSE during this 7-day period, cell clusters were smaller in diameter and very few donut-shaped clusters were observed at day 7 (Figure 2; for quantification see Figure 4A and B). Together these data demonstrate that CSE led to reduced sphere size and inhibited the self-assembly of Caco-2 cells to form single-lumen spheroids.

**Cigarette smoke extract impairs the de novo establishment of epithelial cell polarity**

Epithelial morphogenesis is critically dependent on the ability of the cells to polarize, that is, to develop apical and basolateral plasma membrane domains bordered by tight junctions \(^{41}\), and to orientate their polarity with respect to the extracellular matrix-containing environment \(^{42,43}\). To investigate the effect of CSE on cell polarity and tight junctions, Caco-2 cells were cultured for 7 days in normal 3D culture medium or normal 3D culture medium supplemented with CSE, fixed with PFA and processed for fluorescence (immuno)labeling to visualize the apical actin network, the tight junction protein ZO-1 and cell nuclei.

Most cells cultured in normal culture medium formed single lumen-forming spheroids consisting of a monolayer of cells (evidenced by nuclei staining; Figure 3A). Actin was predominantly present at the lumen surface of the cells (Figure 3A), which reflects the presence of actin-dense apical microvilli and apical surface polarity. ZO-1 appeared predominantly with a punctate pattern at the apex of the lateral plasma membrane facing the lumen (Figure 3A), which is in agreement with the position of the tight junctions \(^{36,38}\). In contrast, cells cultured in medium
Figure 3. Cigarette smoke extract impairs the *de novo* establishment of epithelial Caco-2 cell polarity and morphogenesis. A-B) Labeling pattern of nuclei (blue), actin (red) and ZO-1 (green) in Caco-2 spheroids as formed after 7 days in 3D culture in normal culture medium (A) and in normal culture medium supplemented with CSE (B). C-D) Labeling pattern of nuclei (blue), actin (red) and laminin (green) in Caco-2 spheroids as formed after 7 days in 3D culture in normal culture medium (C) and in normal culture medium supplemented with CSE (D). Asterisks indicate the location of the apical lumen. Bars: 30 um.
supplemented with CSE showed impaired cell polarity and lumen formation (Figure 3B and 4A). Cell nuclei staining revealed that the cells had failed to assemble in monolayers (c.f., Figure 2) and, instead, developed into solid clusters (Figure 3B) of smaller size (Figure 4B). Actin labeling was predominantly observed at the culture medium-facing plasma membrane as well as at the lateral side of the cells (Figure 3B). ZO-1 appeared to be randomly distributed along the cell surface without a clear polarized localization (Figure 3B). In some cases, the cells showed a punctate ZO-1 staining at the lateral sides of the cells, but facing the surrounding medium (Figure 3B, arrowhead), indicative of misoriented cell polarity. Quantification of the data demonstrated that CSE exposure caused a decrease (~50 and ~80% with 7.5 and 10% CSE, respectively) in the percentage of single lumen-forming Caco-2 spheroids (Figure 4A) and in cell cluster size (Figure 4B).

In previous studies it has been shown that the recruitment of laminin, which is a major basement membrane component present in matrigel, to the cell surface is an essential step in epithelial cell polarity orientation and lumen formation in 3D culture \cite{42,44}. We observed predominant laminin accumulation around the polarized lumen-forming Caco-2 clusters under control conditions (Figure 3C), as well as around the non-polarized non-lumen-forming Caco-2 cell clusters exposed to CSE (Figure 3D). This indicates that CSE exposure does not inhibit the recruitment of laminin to the epithelial cell surface. Together, these data indicate that CSE exposure impairs de novo development of Caco-2 cell polarity and tight junctions downstream of laminin recruitment, resulting in defective epithelial morphogenesis.
Figure 4. Effect of NAC and BSO on cigarette smoke extract-induced impairment of Caco-2 epithelial cell polarity and morphogenesis. A) Effects of various treatments (see text) on the percentage of single lumen-forming spheroids in comparison to the control condition (7 days 3D culture in normal culture medium, set to 1); B) Effects of various treatments (see text) on cell cluster size in comparison to the control condition (7 days 3D culture in normal culture medium, set to 1); C) Effects of pre-exposition to CSE and/or NAC (see text) on the percentage of single lumen-forming spheroids in comparison to the control condition (non-pre-exposed cells, set to 1); D) Effects of pre-exposition to CSE and/or NAC (see text) on cell cluster size in comparison to the control condition (non-pre-exposed cells, set to 1); E) Effects of various treatment on intracellular glutathione levels. Continuous data (i.e. size and glutathione expression are presented as (normalized) mean ± SD of at least three independent experiments, dichotomous data (i.e. spheroid formation), is represented as normalized fraction, error bars are ranges.
The inhibitory effect of cigarette smoke extract on epithelial cell polarity and morphogenesis shows penetrance to next generations

In order to determine the (ir)reversibility of the effects of CSE on Caco-2 cell polarization and morphogenesis, we pre-exposed Caco-2 cells to CSE (or control culture medium) for 24 h and subsequently cultivated the cells in 3D in the absence of CSE for 7 days. The cells were then fixed and (immuno)labeled to visualize nuclei, actin and ZO-1, and the percentage of single lumen-forming spheroids was determined. As shown in Figure 4C, CSE-pre-exposed cells showed impaired epithelial polarization and morphogenesis when subsequently grown in 3D culture medium in the absence of CSE, similar to non-pre-exposed cells that were grown in 3D culture medium in the presence of CSE. Notably, CSE pre-exposure did not significantly reduce cell cluster size in next generation cells grown in the absence of CSE (Figure 4D). These data demonstrate the penetrance of the inhibitory effect of CSE on Caco-2 cell polarization and morphogenesis to next generations of cells, which was independent of cell growth.

N-acetyl-L-Cysteine neutralizes the inhibitory effects of cigarette smoke extract on cell growth, cell polarity and morphogenesis

Cigarette smoke extract contains many compounds that are toxic for cells. These include among others reactive oxygen species and potential carcinogens, such as polycyclic aromatic hydrocarbons. Exposure of Caco-2 cells to CSE did not lead to cell death (as analyzed by lactate dehydrogenase leakage tests; data not shown). Many cells, including Caco-2 cells, produce glutathione, a low-molecular-weight thiol, in order to detoxify toxic compounds. Exposure of Caco-2 cells to CSE resulted in increased (~2-fold) levels of intracellular glutathione when compared to cells that were grown in normal culture medium.
(Figure 4D). When Caco-2 cells were grown in 3D culture medium supplemented with CSE together with the glutathione synthesis inhibitor L-buthionine-S,R-sulfoximine (BSO) \(^47\), which completely abolished glutathione production in the cells (Figure 4E), no cells were found attached to the culture plate after 24 h (data not shown), which is indicative for impaired cell viability. When Caco-2 cells were grown in 3D culture medium supplemented with only BSO, normal development of epithelial cell polarity and morphogenesis was observed, indistinguishable to cells cultured in normal medium (Figure 4A, B). These data suggest that CSE stimulated the production of glutathione and glutathione synthesis was required for the cells to survive and form cell clusters in the presence of CSE. We hypothesized that the synthesis of glutathione was sufficient to maintain cell survival when exposed to CSE, but insufficient to prevent the CSE-mediated inhibition of epithelial sphere size, polarity development and morphogenesis. Therefore, we included the glutathione precursor N-acetyl-L-cysteine (NAC) in the CSE-containing culture medium. NAC completely abolished the inhibitory effects of CSE. Thus, Caco-2 cells when cultured in CSE-containing medium supplemented with NAC developed normal-sized single lumen-forming spheroids, similar to cells cultured in normal medium only (Figure 4A). Surprisingly, however, the addition of NAC did not result in increased intracellular glutathione levels (Figure 4D), whereas the neutralizing effect of NAC was abolished in the presence of the glutathione synthesis inhibitor BSO (Figure 4A). This suggested that the neutralizing effect of NAC was via a glutathione-independent mechanism, yet requiring endogenous (i.e., NAC-independent) glutathione synthesis. Interestingly, whereas combined exposure of cells to CSE and BSO inhibited cell viability (see above), the combined exposure of cells to CSE, BSO and NAC did not,
which also indicates a glutathione-independent effect of NAC treatment. Further, in the presence of NAC, glutathione synthesis was not essential to allow cell survival while glutathione synthesis was essential but not sufficient to prevent the inhibition of epithelial cell growth, polarity development and morphogenesis in response to CSE. Interestingly, NAC was not able to restore the polarity and morphogenesis phenotype of CSE-pre-exposed cells (Figure 4C and D), indicating that NAC was required at the time of CSE exposure to neutralize the effects of CSE.

Discussion
The ability of intestinal epithelial cells to self-assemble and form polarized monolayers in conjunction with basement membrane remodeling is a crucial process in the generation of epithelial tissue architecture and epithelial cell homeostasis, including the wound healing response. Here, we show for the first time that cigarette smoke extract directly perturbs the de novo multi-dimensional organization of intestinal epithelial cells in 3D culture, whereas no effect is observed in pre-formed intestinal epithelial cultures. As part of the underlying mechanism, we demonstrate that cigarette smoke impairs cell growth and the de novo development of intestinal epithelial cell polarity (i.e., the segregation of apical and basolateral plasma membrane domains bordered by tight junctions), which is an essential step in the 3D organization of columnar epithelial cells in vitro and in vivo 29.

Previous studies have suggested that cigarette smoke may exert its negative effects on intestinal epithelium homeostasis indirectly via altering the immune system or microbiota 17. While not excluding these possibilities, our study now indicates that also direct effects of cigarette smoke components on the intestinal epithelial cells that interfere with epithelial morphogenesis should be considered.

The precise molecular pathways via which CSE
impaired Caco-2 cell polarity development remains to be elucidated. CSE impairs epithelial sphere size: after 7 days of CSE exposure the size of cell clusters was similar to that of 3 day-old non-exposed cell clusters. Three day-old non-exposed Caco-2 cells already polarize and organize into single lumen-forming spheroids (our unpublished data). Therefore, the reduced size of CSE-exposed cell clusters, as such, is not likely to underlie the cell polarity defects. This is further supported by the notion that the effects of CSE on cluster size and morphogenesis did not follow the same trend as a function of CSE dose (Figure 4A, B). Nevertheless, as cell growth and differentiation are intimately linked processes, it is possible that the reduced growth rate in CSE-exposed cells affects cell polarity development and morphogenesis.

Intestinal epithelial cell differentiation and polarity development depends on cell-cell interactions mediated by adherens junctions and tight junctions, although the necessity for cell-cell interactions for intestinal epithelial cell polarity development has been debated. In airway epithelial cells, exposure to smoke-concentrated medium resulted in loss of cell polarity as a result of perturbed function of p120-catenin function, a protein that plays a role in maintaining the integrity of adherens and tight junctions. Our data demonstrate that ZO-1 is mislocalized in CSE-exposed intestinal epithelial cells, and ZO-1 has been reported to couple the assembly of tight junctions to adherens junctions. Further studies are needed to investigate whether defective adherens and tight junction-mediated cell-cell adhesions underlie the CSE-mediated cell polarization defects in intestinal epithelial cells as reported in this study.

Notably, the inhibitory effects of CSE on cell polarization and morphogenesis was passed on to next generation of cells, even when to were no longer exposed to CSE. Given that smoking has
been demonstrated to induce epigenetic changes \cite{56,57}, and that epithelial cell organization has been linked to epigenetic regulators \cite{58,59}, it is conceivable that epigenetic changes underlie the polarization and morphogenesis defects caused by CSE. While the effects of cigarette smoking on epigenetic modifications in blood cells has been investigated \cite{56}, the effects of smoking on epigenetic modifications in the intestinal epithelium remain unknown. The ability of the intestinal epithelial cells to (re)establish cell polarity and (re)organize themselves into polarized monolayers in response to cues from the extracellular matrix (that is, the basement membrane) is a crucial step in epithelial repair processes \cite{60}. Loss of epithelial cell polarity and consequent defects in multicellular organization has also been associated with the development of cancer \cite{58,61,62}, and cigarette smoking is a risk factor for the development of colorectal cancer. Based on our results we speculate that, in vivo, exposure to cigarette smoke components perturbs repair processes in response to epithelial damage or lesions (such as occur in inflammatory bowel disease) by impairing the ability of intestinal epithelial cells to repolarize and reassemble into monolayers, thereby preventing disease remission and eventually contributing to aberrant organization of the intestinal epithelium and onset of polyp formation and/or carcinogenesis. In this regard it is of interest that especially Crohn’s ileitis is worsened by smoking, and that the ileum is also the site where the first lesions in Crohn’s disease are found \cite{12}. It is tempting to extrapolate the results of this study to other polarized tissues. Indeed, cigarette smoking is also a significant risk factor for intraoral epithelial wound healing after oral surgery \cite{63}, corneal epithelial wound healing \cite{64}, for lung epithelial wound healing \cite{65,66} and for the outcome of plastic surgery patients \cite{67,68}. Possibly, cigarette smoke-induced defects in epithelial cell polarity development
Effects of interferon-gamma and energy metabolism pathways on intestinal epithelial gland morphogenesis in three-dimensional culture

and morphogenesis underlie smoking-associated impaired wound healing also in these typically polarized tissues. Our preliminary data indicate that CSE has similar effects on morphogenesis of MDCK cells, which are tubular kidney epithelial cells (our unpublished results), supports this possibility and warrant further studies. Importantly, the inhibitory effects of cigarette smoke extract on intestinal epithelial cell polarity development and morphogenesis were efficiently neutralized by NAC. Interestingly, NAC-involving therapy was reported to accelerate mucosal healing in a rodent model of colitis. However, the mechanisms by which NAC exerts its beneficial effects on intestinal mucosal healing remain elusive. Whereas NAC is typically used as a precursor for glutathione synthesis, we did not observe elevated levels of intracellular glutathione in NAC-treated Caco-2 cells. Yet, the neutralizing effect of NAC was completely abolished by the glutathione synthesis inhibitor BSO. These data suggest that the neutralizing effect of NAC was not through NAC-stimulated glutathione synthesis, but critically dependent on endogenous (that is, non-NAC-mediated) glutathione synthesis. Of interest in this respect, NAC was reported to promote neuronal PC12 cell survival independently of glutathione but via stimulating transcriptional activity. Moreover, NAC was recently demonstrated to stimulate mammalian target of rapamycin (mTOR)-mediated protein synthesis in intestinal epithelial IPEC cells independent of glutathione synthesis. Given the role of mTOR signaling in polarity of Sertoli cells, further studies are warranted to explore the role of mTOR in NAC-mediated neutralization of CSE-mediated impairment of intestinal epithelial polarity development and morphogenesis. Our data provide further insight into the cell biological mechanisms that may underlie beneficial effects of NAC in the clinical treatment of smoking-related intestinal
disorders. The cigarette smoke component(s) that are responsible for the detrimental effects on 3D intestinal epithelial cell morphogenesis remain to be determined. Cigarette smoke contains free radicals and many carcinogens, including polycyclic aromatic hydrocarbons. In a study that examined the influence of functional glutathione S-transferases (GST) gene polymorphisms on the association between smoking and colorectal cancer, Koh and colleagues reported a role for GST enzymes in the detoxification of colorectal carcinogens in tobacco smoke. We did not obtain evidence of CSE-induced production of reactive oxygen species in the Caco-2 cell spheroids (our unpublished results). Moreover, menadione, a synthetic compound also known as 2-Methyl-1,4-naphthoquinone or vitamin K3 that induces reactive oxygen species, did not mimic the effects of cigarette smoke extract. Moreover, the effects of cigarette smoke extract were not efficiently prevented by the reactive oxygen species inhibitor resveratrol (our unpublished results). Given the clear requirement for glutathione synthesis for the neutralizing effect of NAC in our experiments, it is conceivable that cigarette smoke components that are substrates for GST are responsible for the effects of CSE on intestinal epithelial morphogenesis as demonstrated in this study, and possibly involved in the \textit{in vivo} etiology of smoking-related intestinal epithelium dysfunction.
Effects of interferon-gamma and energy metabolism pathways on intestinal epithelial gland morphogenesis in three-dimensional culture

References

1. Heide F van der, Dijkstra A, Albersnagel FA, et al. Active and passive smoking behaviour and cessation plans of patients with Crohn's disease and ulcerative colitis. J Crohns Colitis 2010;4:125–131.

2. Heide F van der, Dijkstra A, Weersma RK, et al. Effects of active and passive smoking on disease course of Crohn's disease and ulcerative colitis. Inflamm. Bowel Dis. 2009;15:1199–1207.

3. Salim SY, Söderholm JD. Importance of disrupted intestinal barrier in inflammatory bowel diseases. Inflamm. Bowel Dis. 2011;17:362–381.

4. Odenwald MA, Turner JR. Intestinal permeability defects: is it time to treat? Clin. Gastroenterol. Hepatol. 2013;11:1075–1083.

5. Atreya R, Neurath MF. IBD pathogenesis in 2014: Molecular pathways controlling barrier function in IBD. Nat Rev Gastroenterol Hepatol 2015;12:67–68.

6. Rutten EPA, Lenaerts K, Buurman WA, et al. Disturbed intestinal integrity in patients with COPD: effects of activities of daily living. Chest 2014;145:245–252.

7. Li H, Wu Q, Xu L, et al. Increased oxidative stress and disrupted small intestinal tight junctions in cigarette smoke-exposed rats. Mol Med Rep 2015;11:4639–4644.

8. Huxley RR, Ansary-Moghaddam A, Clifton P, et al. The impact of dietary and lifestyle risk factors on risk of colorectal cancer: a quantitative overview of the epidemiological evidence. Int. J. Cancer 2009;125:171–180.

9. Koh W-P, Nelson HH, Yuan J-M, et al. Glutathione S-transferase (GST) gene polymorphisms, cigarette smoking and colorectal cancer risk among Chinese in Singapore. Carcinogenesis 2011;32:1507–1511.

10. Qazi TM, O'Brien MJ, Farraye FA, et al. Epidemiology of goblet cell and microvesicular hyperplastic polyps. Am. J. Gastroenterol. 2014;109:1922–1932.

11. Zuo L, Li Y, Wang H, et al. Cigarette smoking is associated with intestinal barrier dysfunction in the small intestine.
but not in the large intestine of mice. J Crohns Colitis 2014;8:1710–1722.

12. Verschuere S, Bracke KR, Demoor T, et al. Cigarette smoking alters epithelial apoptosis and immune composition in murine GALT. Lab. Invest. 2011;91:1056–1067.

13. Verschuere S, Allais L, Bracke KR, et al. Cigarette smoke and the terminal ileum: increased autophagy in murine follicle-associated epithelium and Peyer’s patches. Histochem. Cell Biol. 2012;137:293–301.

14. Raju SV, Jackson PL, Courville CA, et al. Cigarette smoke induces systemic defects in cystic fibrosis transmembrane conductance regulator function. Am. J. Respir. Crit. Care Med. 2013;188:1321–1330.

15. Wang H, Zhao J-X, Hu N, et al. Side-stream smoking reduces intestinal inflammation and increases expression of tight junction proteins. World J. Gastroenterol. 2012;18:2180–2187.

16. Sun YP, Wang HH, He Q, et al. Effect of passive cigarette smoking on colonic alpha7-nicotinic acetylcholine receptors in TNBS-induced colitis in rats. Digestion 2007;76:181–187.

17. Parkes GC, Whelan K, Lindsay JO. Smoking in inflammatory bowel disease: impact on disease course and insights into the aetiology of its effect. J Crohns Colitis 2014;8:717–725.

18. Bergeron V, Grondin V, Rajca S, et al. Current smoking differentially affects blood mononuclear cells from patients with Crohn’s disease and ulcerative colitis: relevance to its adverse role in the disease. Inflamm. Bowel Dis. 2012;18:1101–1111.

19. Tomoda K, Kubo K, Asahara T, et al. Cigarette smoke decreases organic acids levels and population of bifidobacterium in the caecum of rats. J Toxicol Sci 2011;36:261–266.

20. Benjamin JL, Hedin CRH, Koutsoumpas A, et al. Smokers with active Crohn’s disease have a clinically relevant dysbiosis of the gastrointestinal microbiota. Inflamm. Bowel Dis. 2012;18:1092–1100.

21. Biedermann L, Zeitz J, Mwinyi J, et al. Smoking cessation induces profound changes in the composition of the intestinal microbiota in humans. PLoS ONE 2013;8:e59260.
Effects of interferon-gamma and energy metabolism pathways on intestinal epithelial gland morphogenesis in three-dimensional culture

22. Biedermann L, Brülisauer K, Zeitz J, et al. Smoking cessation alters intestinal microbiota: insights from quantitative investigations on human fecal samples using FISH. Inflamm. Bowel Dis. 2014;20:1496–1501.

23. Aldhous MC, Soo K, Stark LA, et al. Cigarette smoke extract (CSE) delays NOD2 expression and affects NOD2/RIPK2 interactions in intestinal epithelial cells. PLoS ONE 2011;6:e24715.

24. Hickman JA, Graeser R, Hoogt R de, et al. Three-dimensional models of cancer for pharmacology and cancer cell biology: capturing tumor complexity in vitro/ex vivo. Biotechnol J 2014;9:1115–1128.

25. Horning JL, Sahoo SK, Vijayaraghavalu S, et al. 3-D tumor model for in vitro evaluation of anticancer drugs. Mol. Pharm. 2008;5:849–862.

26. Sambale F, Lavrentieva A, Stahl F, et al. Three dimensional spheroid cell culture for nanoparticle safety testing. J. Biotechnol. 2015;205:120–129.

27. Smalley KS, Lioni M, Noma K, et al. In vitro three-dimensional tumor microenvironment models for anticancer drug discovery. Expert Opin Drug Discov 2008;3:1–10.

28. Bryant DM, Mostov KE. From cells to organs: building polarized tissue. Nat. Rev. Mol. Cell Biol. 2008;9:887–901.

29. Roignot J, Peng X, Mostov K. Polarity in mammalian epithelial morphogenesis. Cold Spring Harb Perspect Biol 2013;5.

30. Shamir ER, Ewald AJ. Three-dimensional organotypic culture: experimental models of mammalian biology and disease. Nat. Rev. Mol. Cell Biol. 2014;15:647–664.

31. O’Brien LE, Zegers MMP, Mostov KE. Opinion: Building epithelial architecture: insights from three-dimensional culture models. Nat. Rev. Mol. Cell Biol. 2002;3:531–537.

32. Antoni D, Burckel H, Josset E, et al. Three-dimensional cell culture: a breakthrough in vivo. Int J Mol Sci 2015;16:5517–5527.

33. Tsunoda T, Takashima Y, Fujimoto T, et al. Three-dimensionally specific inhibition of DNA repair-related genes by activated KRAS in colon crypt model. Neoplasia 2010;12:397–404.
34. Jaffe AB, Kaji N, Durgan J, et al. Cdc42 controls spindle orientation to position the apical surface during epithelial morphogenesis. J. Cell Biol. 2008;183:625–633.

35. Durgan J, Kaji N, Jin D, et al. Par6B and atypical PKC regulate mitotic spindle orientation during epithelial morphogenesis. J. Biol. Chem. 2011;286:12461–12474.

36. Juuti-Uusitalo K, Klunder LJ, Sjollema KA, et al. Differential effects of TNF (TNFSF2) and IFN-γ on intestinal epithelial cell morphogenesis and barrier function in three-dimensional culture. PLoS ONE 2011;6:e22967.

37. Elamin E, Masclee A, Juuti-Uusitalo K, et al. Fatty acid ethyl esters induce intestinal epithelial barrier dysfunction via a reactive oxygen species-dependent mechanism in a three-dimensional cell culture model. PLoS ONE 2013;8:e58561.

38. Elamin E, Jonkers D, Juuti-Uusitalo K, et al. Effects of ethanol and acetaldehyde on tight junction integrity: in vitro study in a three dimensional intestinal epithelial cell culture model. PLoS ONE 2012;7:e35008.

39. Toorn M van der, Rezayat D, Kauffman HF, et al. Lipid-soluble components in cigarette smoke induce mitochondrial production of reactive oxygen species in lung epithelial cells. Am. J. Physiol. Lung Cell Mol. Physiol. 2009;297:L109–114.

40. Gonzalez-Mariscal L, Contreras RG, Bolívar JJ, et al. Role of calcium in tight junction formation between epithelial cells. Am. J. Physiol. 1990;259:C978–986.

41. Bryant DM, Datta A, Rodríguez-Fraticelli AE, et al. A molecular network for de novo generation of the apical surface and lumen. Nat. Cell Biol. 2010;12:1035–1045.

42. Bryant DM, Roignot J, Datta A, et al. A molecular switch for the orientation of epithelial cell polarization. Dev. Cell 2014;31:171–187.

43. Overeem AW, Bryant DM, IJzendoorn SCD van. Mechanisms of apical-basal axis orientation and epithelial lumen positioning. Trends Cell Biol. 2015;25:476–485.

44. O’Brien LE, Jou TS, Pollack AL, et al. Rac1 orientates epithelial apical polarity through effects on basolateral laminin assembly. Nat. Cell Biol. 2001;3:831–838.

45. De Angelis I, Rossi L, Pedersen JZ, et al. Metabolism of furazolidone: alternative pathways and modes of toxicity in
Effects of interferon-gamma and energy metabolism pathways on intestinal epithelial gland morphogenesis in three-dimensional culture

different cell lines. Xenobiotica 1999;29:1157–1169.

46. Usta M, Wortelboer HM, Vervoort J, et al. Human glutathione S-transferase-mediated glutathione conjugation of curcumin and efflux of these conjugates in Caco-2 cells. Chem. Res. Toxicol. 2007;20:1895–1902.

47. Griffith OW, Meister A. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). J. Biol. Chem. 1979;254:7558–7560.

48. Laprise P, Chailler P, Houde M, et al. Phosphatidylinositol 3-kinase controls human intestinal epithelial cell differentiation by promoting adherens junction assembly and p38 MAPK activation. J. Biol. Chem. 2002;277:8226–8234.

49. Sääf AM, Halbleib JM, Chen X, et al. Parallels between global transcriptional programs of polarizing Caco-2 intestinal epithelial cells in vitro and gene expression programs in normal colon and colon cancer. Mol. Biol. Cell 2007;18:4245–4260.

50. Halbleib JM, Sääf AM, Brown PO, et al. Transcriptional modulation of genes encoding structural characteristics of differentiating enterocytes during development of a polarized epithelium in vitro. Mol. Biol. Cell 2007;18:4261–4278.

51. Baas AF, Kuipers J, Wel NN van der, et al. Complete polarization of single intestinal epithelial cells upon activation of LKB1 by STRAD. Cell 2004;116:457–466.

52. Gon H, Fumoto K, Ku Y, et al. Wnt5a signaling promotes apical and basolateral polarization of single epithelial cells. Mol. Biol. Cell 2013;24:3764–3774.

53. Zhang L, Gallup M, Zlock L, et al. Cigarette smoke disrupts the integrity of airway adherens junctions through the aberrant interaction of p120-catenin with the cytoplasmic tail of MUC1. J. Pathol. 2013;229:74–86.

54. Gu C, Liu M, Zhao T, et al. Protective role of p120-catenin in maintaining the integrity of adherens and tight junctions in ventilator-induced lung injury. Respir. Res. 2015;16:58.

55. Maiers JL, Peng X, Fanning AS, et al. ZO-1 recruitment to a-catenin--a novel mechanism for coupling the assembly of tight junctions to adherens junctions. J. Cell. Sci. 2013;126:3904–3915.
56. Tsaprouni LG, Yang T-P, Bell J, et al. Cigarette smoking reduces DNA methylation levels at multiple genomic loci but the effect is partially reversible upon cessation. Epigenetics 2014;9:1382–1396.

57. Zeilinger S, Kühnel B, Klopp N, et al. Tobacco smoking leads to extensive genome-wide changes in DNA methylation. PLoS ONE 2013;8:e63812.

58. Bunker BD, Nellimoottil TT, Boileau RM, et al. The transcriptional response to tumorigenic polarity loss in Drosophila. Elife 2015;4.

59. Mazzolini R, Dopeso H, Mateo-Lozano S, et al. Brush border myosin Ia has tumor suppressor activity in the intestine. Proc. Natl. Acad. Sci. U.S.A. 2012;109:1530–1535.

60. King RS, Newmark PA. The cell biology of regeneration. J. Cell Biol. 2012;196:553–562.

61. Hinck L, Näthke I. Changes in cell and tissue organization in cancer of the breast and colon. Curr. Opin. Cell Biol. 2014;26:87–95.

62. Halaaoui R, McCaffrey L. Rewiring cell polarity signaling in cancer. Oncogene 2015;34:939–950.

63. Jones JK, Triplett RG. The relationship of cigarette smoking to impaired intraoral wound healing: a review of evidence and implications for patient care. J. Oral Maxillofac. Surg. 1992;50:237–239; discussion 239–240.

64. Jetton JA, Ding K, Kim Y, et al. Effects of tobacco smoking on human corneal wound healing. Cornea 2014;33:453–456.

65. Heijink IH, Brandenburg SM, Postma DS, et al. Cigarette smoke impairs airway epithelial barrier function and cell-cell contact recovery. Eur. Respir. J. 2012;39:419–428.

66. Rennard SI, Togo S, Holz O. Cigarette smoke inhibits alveolar repair: a mechanism for the development of emphysema. Proc Am Thorac Soc 2006;3:703–708.

67. Netscher DT, Clamon J. Smoking: adverse effects on outcomes for plastic surgical patients. Plast Surg Nurs 1994;14:205–210.

68. Pluvy I, Panouillères M, Garrido I, et al. Smoking and plastic surgery, part II. Clinical implications: a systematic
Effects of interferon-gamma and energy metabolism pathways on intestinal epithelial gland morphogenesis in three-dimensional culture

review with meta-analysis. Ann Chir Plast Esthet 2015;60:e15–49.

69. Siddiqui A, Ancha H, Tedesco D, et al. Antioxidant therapy with N-acetylcysteine plus mesalamine accelerates mucosal healing in a rodent model of colitis. Dig. Dis. Sci. 2006;51:698–705.

70. Yan CY, Ferrari G, Greene LA. N-acetylcysteine-promoted survival of PC12 cells is glutathione-independent but transcription-dependent. J. Biol. Chem. 1995;270:26827–26832.

71. Yi D, Hou Y, Wang L, et al. N-acetylcysteine stimulates protein synthesis in enterocytes independently of glutathione synthesis. Amino Acids 2015.

72. Tanwar PS, Kaneko-Tarui T, Zhang L, et al. Altered LKB1/AMPK/TSC1/TSC2/mTOR signaling causes disruption of Sertoli cell polarity and spermatogenesis. Hum. Mol. Genet. 2012;21:4394–4405.

73. Sobus SL, Warren GW. The biologic effects of cigarette smoke on cancer cells. Cancer 2014;120:3617–3626.
Loss of intestinal Myosin Vb function impairs Claudin-1 trafficking in microvillus inclusion disease