Chapter 4

Effects of interferon-gamma and energy metabolism pathways on intestinal epithelial gland morphogenesis in three-dimensional culture

Leon J. Klunder¹, Tjasso Blokzijl², 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

Manuscript in preparation
Abstract
Energy metabolism plays an important role in intestinal epithelial homeostasis and colon cancer. Three-dimensional (3D) intestinal epithelial cell cultures have recently been developed as an in vitro model to study intestinal epithelial and colon cancer cell homeostasis and colorectal glandular morphogenesis, but the role of energy metabolism in these model systems has not been addressed. Here, we investigated the role of two energy metabolic pathways, mitochondrial oxidative phosphorylation and glycolysis, in apical-basal polarization, proliferation and morphogenesis of isogenic colon carcinoma Caco-2 cells in 3D culture under normal and interferon gamma (IFNγ)-stimulated conditions. Our data demonstrate that selective pharmacological inhibition of either oxidative phosphorylation with oligomycin or glycolysis with 2-deoxy-D-glucose impaired, but not completely blocked cell proliferation, resulting in cell clusters of smaller size. Inhibition of either oxidative phosphorylation or glycolysis did not affect apical-basal polarity development and glandular morphogenesis, as evidenced by the formation of cell clusters consisting of single layers of polarized cells surrounding a central apical lumen. Interestingly, treatment of the cells with the pro-inflammatory cytokine IFNγ stimulated the rate of cell proliferation without affecting cell polarity and morphogenesis. IFNγ-stimulated proliferation was dependent on glycolysis, but not oxidative phosphorylation. Our data demonstrate 1) that distinct (stimulus-triggered) cellular processes that are relevant to 3D intestinal epithelial cell morphogenesis differently utilize and depend on oxidative phosphorylation and glycolysis, 2) that under conditions of energy restriction proliferation, but not polarity and morphogenesis is affected, and 3) that targeting glycolysis may neutralize IFNγ-inflicted effects on intestinal epithelial (cancer) cell behavior.
**Introduction**

Intestinal epithelial cells make up the innermost layer of the intestinal tract. They are organized as a monolayer of polarized cells with their apical surface domains facing the intestine lumen and their basal surface domains facing the basement membrane and lamina propria. Cell-cell interactions provide for the barrier function of the intestinal epithelium. Intestinal epithelial cells arise from stem cells that reside in the crypts. These stem cells first give rise to a transient pool of highly proliferative intestinal epithelial cells that then differentiate and polarize as they migrate towards the villus tip, where they are finally extruded from the monolayer, all in the course of 3-4 days. This process of intestinal epithelial cell morphogenesis is the result of a dynamic integration of key cellular processes, such as migration, proliferation, differentiation and polarization, acting in conjunction with cues from the extracellular environment to maintain epithelial order \(^1,2\). Defective intestinal epithelial morphogenesis can lead to the disruption of the functional architecture of the intestinal epithelium and has been associated with epithelial disorder, *e.g.*, dysplasia, polyposis \(^3,4\) and the development of colon cancer \(^2\).

Key epithelial morphogenetic processes, such as proliferation, differentiation and cell polarity are tightly linked to nutrient status and cellular energy metabolism. The two main energy metabolic pathways in mammalian cells are mitochondrial oxidative phosphorylation and (aerobic) glycolysis. During oxidative phosphorylation, adenosine triphosphate (ATP) is formed following the oxidation of nutrients in mitochondria. During glycolysis, glucose is converted into pyruvate thereby generating ATP. How cellular processes that are associated with intestinal epithelial morphogenesis depend on these energy metabolism pathways is not known.
Metabolic fingerprinting revealed a metabolic crypt-to-villus gradient in the mouse small intestine. A transient upregulation of the expression of enolase, a key protein involved in glycolysis, was observed during the differentiation process of intestinal epithelial Caco-2 cells. Also primary rat hepatocytes, which are the main epithelial cells in the liver, were reported to shift from mitochondrial oxidative phosphorylation to glycolysis as part of the cell polarization process. These reports suggest that epithelial cells can switch between energy metabolism pathways during their differentiation process, thereby conceivably contributing to epithelial morphogenesis.

The putative link between energy metabolism pathways and intestinal epithelial morphogenesis is of particular interest in the context of colon cancer development. Colon cancer, like most cancers, are derived from disordered epithelial cells and arises as a consequence of progressive changes from normal epithelial cells through polyp to tumor. Cancer cells, including colon cancer cells, typically have an energy metabolism shifted from oxidative phosphorylation to aerobic glycolysis (Warburg effect) in order to accommodate their enhanced proliferation rate. In intestinal epithelial cells of Abhd5 knock-out mice, an increase in aerobic glycolysis and a resultant induction of epithelial to mesenchymal transition was recently shown to promote colon cancer development and progression, suggesting a causal relationship between enhanced glycolysis and cancer development.

The etiology of colon cancer includes both genetic and environmental factors. The risk of developing (colitis-associated) colon cancer is enhanced in individuals with inflammatory bowel disease (IBD), and has been linked to high levels of circulating pro-inflammatory cytokines. Two pro-inflammatory cytokines, tumor necrosis factor-alpha (TNFα) and interferon-gamma (IFNγ), that
are elevated in IBD can bind to receptors at the basal surface domain of intestinal epithelial cells and elicit intracellular signaling pathways that disturb intestinal epithelial monolayer integrity 16–19. Interestingly, TNFα 20–24 and IFNγ 21,24–26 have been reported to stimulate glycolysis in a variety of cell types, including intestinal epithelial cells. The inhibition of specific energy metabolism pathways have been reported to attenuate intestinal epithelial barrier dysfunction induced by IFNγ and TNFα 27, suggesting that energy metabolism pathways play an important role in cytokine-mediated perturbations in intestinal epithelial integrity. Importantly, TNFα and IFNγ were found to perturb intestinal epithelial morphogenesis in a three-dimensional culture system 28, but the involvement of energy metabolism was not addressed. Thus, at least in vitro, a pro-inflammatory environment can give rise to defective glandular morphogenesis of intestinal epithelial cells and alterations in energy metabolism that are characteristic for colon cancer.

Three-dimensional (3D) intestinal epithelial Caco-2 cell cultures have been proven useful as an in vitro model system to study the effects of genetic and environmental factors on colorectal glandular morphogenesis35-40. Caco-2 is a low-invasive 29 human colorectal carcinoma cell line with a tumorigenic phenotype, which is lost and replaced by morphological and biochemical features of intestinal epithelial cells upon differentiation 6,30–33. Caco-2 cells show contact-inhibition of growth and changes in the expression levels of mRNA 30,31,34 and proteins 6,32 associated with their proliferation and differentiation, which are similar to those during enterocyte differentiation in vivo 31. Importantly, when single Caco-2 cells are plated in the presence of basement membrane extract (matrigel), they proliferate and, as a function of time, assemble a basement membrane and self-organize into spheroids that consist of a monolayer of polarized
cells surrounding a central apical lumen, mimicking the architecture of colorectal glands. This culture system was used to study epithelial barrier function, cell polarization and morphogenesis in response to ethanol, acetyl aldehyde, and fatty acid ethyl esters, and to investigate 3D colorectal glandular formation and the disruption of colorectal glandular architecture and morphogenesis as a result of PTEN (phosphatase and tensin homolog deleted on chromosome ten) deficiency. The role of energy metabolism pathways in this 3D model system has, however, not been addressed.

In this study, we have investigated the role of energy metabolism pathways in Caco-2 cell proliferation, differentiation and polarization, and resultant epithelial morphogenesis and glandular architecture development in 3D culture under normal and IFNγ-stimulated conditions.

Materials and Methods

*Three-dimensional Caco-2 cell culture.* Three-dimensional (3D) culture of human Caucasian colon carcinoma (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; 4.5 g/L glucose) 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, plated at a concentration of 1.5×10⁴ cells/ml in wells of a 8-well plate pre-coated with 100% (v/v) Matrigel and cultured for up to 7 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\textsuperscript{28,35,38}.

**Treatment of cells.** In some experiments, cells were grown in 3D culture in medium supplemented with the ATP synthase inhibitor oligomycin A (10 μg/mL) (Sigma-Aldrich, Selze, Germany), 2-deoxy-D-glucose (10 mM) (Sigma-Aldrich) and mannose (2.5 mM) (Sigma-Aldrich), and/or recombinant human interferon-gamma (IFNγ, 100 IU/ml) (Peprotech, Rocky Hill/NJ, USA)\textsuperscript{28} for the indicated time periods.

**Fluorescence labeling, microscopy, and digital image processing.** 3D cell cultures were fixed with 4% PFA at 37°C for 1 h, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) at room temperature for 20 minutes and washed with Hank’s balanced salt solution (HBSS). Filamentous actin was visualized with tetramethylrhodamine (TRITC)-phalloidin (1:1000, Sigma-Aldrich) or TRITC-phalloidin-688 (1:1000, Sigma-Aldrich). Nuclei were visualized with 4’,6-diamidino-2-phenylindole (DAPI; 1:1000). 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. For cluster sizes, the area per cluster was measured using the phalloidin staining.

**Quantitative Polymerase Chain Reaction (qPCR).** RNA was extracted from cells according to the manufacturer’s instructions (Purelink RNA micro kit, Invitrogen, Carlsbad, USA). cDNA was synthesized using poly-dT primer and reverse transcriptase (Invitrogen) following the manufacturers protocol. qRT primers were designed using perlprimer (perlprimer.scourgeforge.net). The ΔCt values of the IFNy-treated group were compared with those for the controls. Primers used
are listed in supplementary Table 1. Reactions were run on a AB StepOnePlus (ThermoFischer scientific, Waltham, USA). Cycling conditions comprised 15 minutes polymerase activation at 95°C followed by 40 cycles at 95°C for 15 sec and 60°C for 30 sec. HMBS was used as an internal control, Ct value for HMBs was subtracted from the Ct value of the target gene (ΔCt).

**Measurement of lactic acid.** Lactate measurements were performed using a Lactate Assay Kit (Megazyme, Wicklow Ireland), according to the manufacturer’s instructions. In brief, cells were seeded in 3D culture as described above in medium supplemented with or without IFNγ. After 7 days, culture medium was harvested and lactate levels were determined spectrophotometrically using a Selectra ProM spectrophotometer (ELITech Clinical Systems) at 37 °C after 5 min. The lactate levels were subsequently normalized to the amount of cells, the normalized value for control was set to 1.

Cells were seeded in 3D culture as described above in medium supplemented with IFN- gamma or not. After 7 days supernatant was taken, and lactate levels were determined spectrophotometrically assessing trough spectrofotometrically assessing the reaction with lactate dehydrogenase, using the Selectra ProM spectrophotometer (ELITech Clinical Systems), measuring at 37°C* after 5 min. The lactate levels were subsequently normalized to the amount of cells and control.

Cells were seeded in 3D culture as described above in medium supplemented with IFN-gamma or not. After 7 days supernatant was taken, and lactate levels were determined spectrophotometrically assessing trough spectrofotometrically assessing the reaction with lactate dehydrogenase, using the Selectra ProM spectrophotometer (ELITech Clinical Systems), measuring at 37°C* after 5 min.
The lactate levels were subsequently normalized to the amount of cells and control
Cells were seeded in 3D culture as described above in medium supplemented with IFN-gamma or not. After 7 days supernatant was taken, and lactate levels were determined spectrophotometrically assessing trough spectrophotometrically assessing the reaction with lactate dehydrogenase, using the Selectra ProM spectrophotometer (ELITech Clinical Systems), measuring at 37°C after 5 min. The lactate levels were subsequently normalized to the amount of cells and control

Mathematical modeling. In order to mathematically estimate the number of cells present in a spheroid of a given diameter, the following formula (formula 1) is used:

\[(4 \pi r^2)/x\]

in which \( r \) is the radius of the spheroid in μm as measured in a cross-sectional confocal microscopy image from the center of the spheroid to the basal plasma membrane, and \( x \) is the average basal surface area of a Caco-2 cell in the spheroid monolayer (set to 100 μm²). In this formula a perfect sphere shape is assumed.

The average cell doubling time during a given culture period is estimated using the following formula (formula 2):

\[d \log^2 / (\log [(4 \pi r^2)/x] - \log [y])\]

in which \( d \) is the duration of the culture in hours, and \( y \) is the number of cells at the start time (typically 1). In this formula it is assumed that spheroids arise clonally from one cell, as has been demonstrated experimentally \(^{28,36,37}\).
The relative difference between the estimated average cell doubling time between two experimental conditions is calculated using the following formula (formula 3):

\[
\frac{(d \cdot \log_2 (\frac{(4\pi n r^2)}{x}) - \log [y])_A}{(d \cdot \log_2 (\frac{(4\pi n r^2)}{x}) - \log [y])_B} \times 100
\]

in which \( A \) and \( B \) represent the two experimental conditions that are compared.

**Statistical analyses.** Fractions of cell clusters with micronuclei as part of total cell clusters, and fractions of lumens with extruded cells as part of total lumens were compared using \( \chi^2 \)-square tests. Cluster sizes were compared using \( t \)-tests.

**Results**

**Glycolysis and oxidative phosphorylation together contribute to Caco-2 cell proliferation in 3D culture**

Mammalian cells are known to utilize two main energy metabolism pathways, oxidative phosphorylation and aerobic glycolysis. In order to investigate to what extent Caco-2 cell growth in 3D culture depends on oxidative phosphorylation, single cells were plated for 3D culture in normal culture medium (control) or in normal culture medium supplemented with oligomycin A at a non-lethal dose. Oligomycin A inhibits H\(^+\)-ATP synthase, an enzyme that is necessary for the oxidative phosphorylation of ADP to ATP, i.e., energy production \(^{41}\). Cells cultured in normal medium (in the absence of oligomycin) developed multicellular spherical clusters with an average diameter of 90 \( \mu m \) over a period of 7 days (Figure 1A). In contrast, when cells were cultured in the presence of oligomycin, the cluster cross-sectional area was significantly reduced by 2-fold (Figure 1B; quantification in Figure 1G).
When the cell clusters were stained with DAPI, which labels DNA and thereby the cell nuclei, it was noted that the number of cells per cluster was reduced in oligomycin-treated cultures (Figure 1E) when compared to non-treated cultures (Figure 1D).

Figure 1. Effects of oligomycin and 2-DG on Caco-2 spheroid development. Caco-2 cells were cultured in 3D culture in normal culture medium (A, D), normal culture medium supplemented with oligomycin A (B, E) or with 2-deoxy-D-glucose (2-DG; C, F) for 7 days, fixed and processed for DAPI staining and microscopical analysis. A-C show bright field images of the spheroids. D-F show fluorescent DAPI staining. G) shows the quantification of the cluster size of control, oligomycin A- and 2-DG-treated cells (normalized to control conditions). H) shows the percentage of spheroids with extruded cells in the spheroid lumen. I) shows the percentage of spheroids with signs of nuclear fragmentation. n.s.: not significant. Bars: 30 µm.
Mathematical modeling (see Materials and Methods) indicates that a 2-fold reduction in spheroid cross-sectional area (with no changes in the basal surface area occupied by a single cell) represents an estimated 2-fold reduction in cell number. No signs of increased cell extrusion into the lumens (quantification in Figure 1G) or apoptosis (i.e., the presence of micronuclei or fragmented nuclei) (Figure 1F) were observed in the 2-DG treated cell clusters. These data suggested that oxidative phosphorylation is utilized by the cells to proliferate, but that additional energy generating metabolic pathways also contribute to proliferation.

We next investigated the role of glycolysis in Caco-2 cell proliferation in 3D culture. For this, cells were plated for 3D culture in normal culture medium (control) or in normal culture medium supplemented with 2-deoxy-D-glucose at a non-lethal dose. 2-deoxy-D-glucose is a modified glucose molecule that cannot undergo glycolysis and acts competitively to inhibit the conversion of natural glucose to pyruvate and the production of ATP from this pathway. In all incubation with 2-DG, mannose was included, which reverts 2-DG-induced endoplasmic reticulum stress, but not ATP depletion (Ramirez-Peinado et al., 2011). When cells were cultured in the presence of 2-DG, the cluster cross-sectional area was significantly reduced with ~50% (Figure 1C) (i.e., an estimated 2-fold reduction in cell number per spheroid; see above) when compared to cell cultured in the absence of 2-DG (Figure 1A; quantification in Figure 1G). DAPI staining revealed a decreased number of cells per cluster in 2-DG-treated cultures (Figure 1F) when compared to non-treated cultures (Figure 1D). No signs of increased cell extrusion into the lumens (quantification Figure 1H) or apoptosis (i.e., the presence of micronuclei or fragmented nuclei) were observed in the treated cell clusters (Figure 1F; quantification in 1I.). These data suggest that glycolysis is utilized by the cells to proliferate. Similar to the experiments in which oxidative phosphorylation was inhibited, glycolysis appears not to be the sole source of energy that is
used by the cells for proliferation. Caco-2 cells did not survive combined treatment with oligomycin A and 2-DG (data not shown). We conclude that glycolysis and oxidative phosphorylation together contribute to Caco-2 cell proliferation in 3D culture.

Caco-2 cell polarity development and 3D glandular morphogenesis does not selectively require glycosylation or oxidative phosphorylation

The development of apical-basal cell polarity development is a crucial step in epithelial morphogenesis, that is, the process in which the cells self-assemble into monolayers surrounding a central apical lumen \(^{42-44}\). Cell polarity development and energy metabolism are interrelated processes \(^{45-47}\). Moreover, it has been reported that liver epithelial cells shift from oxidative phosphorylation to glycolysis as part of the cell polarization process \(^7\). Therefore, we examined the contribution of oxidative phosphorylation and glycolysis to Caco-2 cell polarity development and morphogenesis in 3D culture. For this, Caco-2 cells were, as described above, plated for 3D culture in normal culture medium supplemented or not supplemented with either oligomycin A or 2-DG, . After 7 days, cells were fixed and processed for labeling with TRITC-phalloidin and DAPI. Untreated cells had organized into a single cell layer surrounding a central lumen (Figure 2A; c.f., Figure 1D). Filamentous (F-) actin was predominantly at the lumen-facing surface of the cells (Figure 2B; merged image of DAPI and TRITC-phalloidin in Figure 2C, graph of distribution profile of actin from basal to basal in Figure 2D), which reflects the presence of actin-filled apical microvilli and is indicative for apical-basal cell polarity \(^{28,36,37}\). When cells had been treated with either oligomycin A (Figure 2E-H) or 2-DG (Figure 2I-L), cells displayed a similar organization, albeit in cell clusters of smaller size. Thus, DAPI staining revealed the organization of cells in a single cell layer surrounding a central
Effects of interferon-gamma and energy metabolism pathways on intestinal epithelial gland morphogenesis in three-dimensional culture

Figure 2. Effects of oligomycin and 2-DG on Caco-2 cell polarity and morphogenesis. Caco-2 cells were cultured in 3D culture in normal culture medium (A-D), normal culture medium supplemented with oligomycin A (E-H) or with 2-deoxy-D-glucose (2-DG; I-L) for 7 days, fixed and processed for DAPI (blue) and TRITC-phalloidin (red) staining and microscopical analysis. The graphs in D, H and L depict the distribution profile of actin (red channel) on a line from basal to basal. Bars: 30 µm.

Lumen (Figure 2 G and K), and TRITC-phalloidin staining revealed the predominant expression of F-actin at the lumen-facing cell surfaces (Figure 2F and H, and J and L). These results indicate that Caco-2 cell polarity development and epithelial 3D morphogenesis did not selectively require glycolysis or oxidative metabolism as it was unaffected by the inhibition of either of these two processes. The
results also demonstrate that under conditions of energy restriction cells cut down on proliferation, but maintain their polarized phenotype and normal morphogenesis.

**IFN-γ stimulates Caco-2 cell proliferation in 3D culture without perturbing glandular architecture**

Intestinal epithelial homeostasis is compromised under inflammatory conditions, such as in Crohn’s disease and ulcerative colitis. Several cytokines can bind receptors on the intestinal epithelial cell surface resulting in perturbation of key cellular processes, such as cell-cell adhesion (barrier function), apoptosis and proliferation. Here, we have investigated the effects of IFNγ on Caco-2 cell morphogenesis. For this, the cells were plated for 3D culture in normal culture medium (control) or in normal culture medium supplemented with IFNγ. Cells cultured in normal medium developed multicellular spherical clusters with an average diameter of ~90 μm over a period of 7 days (c.f., Figure 1A and 2A). Staining of the cells with TRITC-phalloidin and DAPI showed that the cell clusters consisted of a single layer of polarized cells surrounding a central apical lumen. When cells were cultured in the presence of IFNγ, the cluster cross-sectional area was significantly increased by 50% (Figure 3B). Note that a 1.5-fold increase in spheroid cross-sectional area indicates a similar 1.5-fold increase in the average number of cells per spheroid (see also above). Staining of the cells with TRITC-phalloidin and DAPI revealed that IFNγ did not perturb Caco-2 cell morphogenesis, as the cell clusters consisted of a single layer of polarized cells surrounding a central apical lumen (Figure 3A). Collectively, these data suggest that IFNγ stimulated the proliferation rate of Caco-2 cells in 3D culture without affecting epithelial cell morphogenesis.
Effects of interferon-gamma and energy metabolism pathways on intestinal epithelial gland morphogenesis in three-dimensional culture

IFNγ-stimulated Caco-2 cell proliferation requires glycolysis and not oxidative phosphorylation.

An increase in cell proliferation requires energy. Given that both oxidative phosphorylation and
glycolysis contributed to Caco-2 cell proliferation in 3D culture (see Figures 1 and 2), we next examined the dependency of IFNγ-stimulated proliferation on each of the energy metabolism pathway. For this, cells were plated for 3D culture in normal culture medium supplemented with either oligomycin A or 2-DG and IFNγ. IFNγ increased the relative cluster size in oligomycin A-treated cells to a similar extend as it did in Caco-2 cells grown in normal conditions (Figure 3B). In contrast, IFNγ failed to stimulate proliferation in 2-DG-treated cultures (Figure 3B). These data suggested that IFNγ-stimulated proliferation of Caco-2 cells specifically required glycolysis. Treatment of cells with IFNγ did not result in increased concentrations of lactic acid in the culture medium (Figure 3C) and did not stimulate the expression of genes that are associated with glycolysis, i.e., gpi, aldoa, pgk1, eno1 and pklr (figure 3D) and hk2, hk3, gck, pgm1, pgm2, galm, pfkl, aldob, aldoc, tpi, pgk, pgam, eno2, eno3 (data not shown) beyond statistical significance, suggesting that the cytokine itself did not stimulate glycolysis.

Discussion

In this study, we have addressed the role of energy metabolism pathways in intestinal epithelial Caco-2 epithelial cell morphogenesis in 3D culture. We found that inhibition of either oxidative phosphorylation of glycolysis resulted in the formation of glandular spheroids of significant smaller size. A reduction in spheroid size could theoretically result from increased apoptosis, increased cell shedding into the apical lumen, or decreased proliferation. However, no micronuclei or fragmented nuclei (indicative for apoptosis) and no increase in the appearance of cells in the spheroids lumen (indicative for enhanced cell extrusion) were observed in oligomycin A- or
Effects of interferon-gamma and energy metabolism pathways on intestinal epithelial gland morphogenesis in three-dimensional culture

2-DG-exposed cultures. By contrast, we observed a reduction in the average number of cells (identified by the number of DAPI-stained nuclei) in oligomycin or 2-DG-exposed Caco-2 spheroids. Together, our data suggest that treatment with oligomycin A or 2-DG resulted in smaller size spheroids via inhibition of cell proliferation and, hence, that oxidative phosphorylation and glycolysis additively contribute to Caco-2 spheroid growth.

The exposure of the cells to IFNγ resulted in the development of glandular spheroids of significantly larger size. We observed no decrease in the number of cells or cell debris in the spheroid lumens, suggesting that the increase in spheroid size was not due to reduced extrusion of cells from the monolayer. In addition, we observed no flattening of the cells surrounding the lumen (which would be expected if apical lumen expansion rather than proliferation was increased), and therefore argues against an increase of trans-epithelial chloride secretion (via the ATP-binding cassette transporter CFTR) as a possible cause of increased spheroid size. These observations, together with the observed increase in the number of cells in the spheroids, suggest that IFNγ stimulates cell proliferation.

The stimulatory effect of IFNγ on cell proliferation and thereby on spheroid size was blocked by 2-DG, but not by oligomycin A, which suggests a specific requirement for glycolysis. IFNγ has been reported to modulate glycolysis in different cell types. For example, in dermal fibroblasts, IFNγ, when combined with TNFα, increased the dependency for cellular energy provision from an oxidative to the glycolytic state. In rheumatoid synovial cells, IFNγ produced a 3-6-fold increase in cellular fructose 2,6-bisphosphate, an indicator of glycolysis. Also in intestinal epithelial HT-19 cells, increased lactate was detected after apoptosis-inducing IFNγ/TNFα treatment, but not in cells that were TNFα-treated without IFNγ preincubation.

In intestinal
epithelial T84 cells, IFNγ stimulated the expression of hypoxia-inducible factor-1 \(^{50}\), the overexpression of which in pancreas adenocarcinoma BxPC3 cells enhanced the expression of pyruvate dehydrogenase kinase 1 and lactate dehydrogenase A, thus facilitating glycolysis \(^{51}\). In glioma cells, by contrast, metabolic gene profiling indicated a suppressed glycolytic pathway upon IFNγ treatment \(^{52}\). In our study, the profiling of metabolic gene expression and lactic acid measurements in the culture medium (note that lactic acid efflux is considered to occur at the basolateral plasma membrane \(^{53}\)) did not provide evidence for IFNγ-stimulated glycolysis. Furthermore, co-treatment of the cells with IFNγ and either 2-DG or oligomycin A did not result in spheroids of smaller size in comparison to treatment of the cells withy 2-DG or oligomycin A alone. This suggests that IFNγ treatment did not render the cells more growth-sensitive to either glycolysis or oxidative phosphorylation, \textit{i.e.}, did not shift the metabolic profile from oxidative phosphorylation to glycolysis or \textit{vice versa}. In support of these \textit{in vitro} results, we found no evidence of a significantly altered expression of glycolysis-related genes between inflamed and non-inflamed colonic tissues from individuals diagnosed with the inflammatory bowel disease ulcerative colitis, despite a clear overall increase in IFNγ expression in the inflamed tissue specimens (data not shown). If IFNγ altered the expression of glycolysis-associated genes, such changes were too small to extend beyond the range of normal variation in our experimental setting. The reason why the proliferation-stimulating effect of IFNγ required glycolysis and not oxidative phosphorylation remains elusive, but may involve the consumption of pyruvate for the production of macromolecular precursors, such as acetyl-CoA for fatty acids, glycolytic intermediates for nonessential amino acids, and ribose for nucleotides, which are necessary to support cell proliferation \(^{54}\).
The processes of cell proliferation on the one hand and of epithelial cell differentiation and polarity development on the other hand are believed to be intimately related. Enhanced proliferation has been proposed to elicit detrimental effects on epithelial morphogenesis and the development of a glandular architecture. Our data revealed that Caco-2 cells proliferation in 3D culture can be substantially reduced (by oligomycin or 2-DG) or enhanced (by IFNγ) without visible deleterious effects on the glandular architecture of the spheroids formed. In order to appreciate the effect of IFNγ at the level of the single cell rather than at the level of the spheroid formed, we used mathematical modeling to estimate the changes in cell doubling time that underlie the change in spheroid size. Using formulas 1-3 as described in the Materials and Methods section, we estimated that IFNγ shortens the average Caco-2 cell doubling time with ~7% (from 21 h in non-treated cultures to 19.6 h in IFNγ-treated cultures). Thus, the pronounced effect on the size of the spheroids formed is the result of a relatively modest effect of IFNγ on the doubling time per cell. The modest reduction in cell cycle time likely falls within the range of equilibrium dynamics, and explains how spheroids of significant larger size can be formed without deleterious effects on their polarized glandular architecture.

The mechanisms by which IFNγ stimulates proliferation and spheroid size remain to be elucidated. IFNγ has been shown to increase the permeability of intestinal epithelial cell monolayers via perturbation of tight junction-mediated cell-cell adhesion, also in three-dimensional intestinal epithelial cell cultures. Functional tight junctions have been implicated in the regulation of proliferation, as specific transcription factors can be sequestered at tight junctions and translocate to the nucleus when tight junctions are perturbed. Therefore, it is conceivable that IFNγ-stimulated
proliferation (this study) is secondary to IFNγ-mediated perturbation of tight junctions. However, treatment of intestinal epithelial cells in 3D culture with TNFα, ethanol or ethanol metabolites similarly perturbed tight junctions, but did not stimulate proliferation, indicating that loss of tight junction function does not automatically lead to enhanced proliferation. The intriguing question can then be raised whether IFNγ-stimulated proliferation (this study) may lead to enhanced permeability and, if so, whether inhibition of glycolysis with 2-DG can neutralize the negative impact of IFNγ on intestinal barrier function without compromising other processes that are required for epithelial morphogenesis.

In conclusion, the results presented in this study demonstrate 1) that distinct (stimulus-triggered) cellular processes that are relevant to 3D intestinal epithelial cell morphogenesis differently utilize and depend on oxidative phosphorylation and glycolysis, 2) that under conditions of energy restriction proliferation, but not polarity and morphogenesis, is affected, and 3) that targeting glycolysis may neutralize IFNγ-inflicted effects on intestinal epithelial (cancer) cell behavior.

References


4. Wiebecke B, Brandts A, Eder M. Epithelial proliferation and morphogenesis of hyperplastic adenomatous and villous
Effects of interferon-gamma and energy metabolism pathways on intestinal epithelial gland morphogenesis in three-dimensional culture


Effects of interferon-gamma and energy metabolism pathways on intestinal epithelial gland morphogenesis in three-dimensional culture


### Supplementary table 1. qPCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPI</td>
<td>5’-TGC CTC CAA GAC CTT TAC TAC C-3’</td>
<td>5’-GGT CAA TTC CAA ACT CCT TCA C-3’</td>
</tr>
<tr>
<td>ALDOA</td>
<td>5’- TGA GTG ACC ACC ACA TCT ACC- 3’</td>
<td>5’- AGG AAG GTG ATC CCA GTG AC -3’</td>
</tr>
<tr>
<td>PGK1</td>
<td>5’-CCA AGT CGG TAG TCC TTA TGA G-3’</td>
<td>5’-AAC AGA ACA TCC TTG CCC AG-3’</td>
</tr>
<tr>
<td>ENO1</td>
<td>5’-CAT GGT GTC TCA TCG TTC GG-3’</td>
<td>5’-TCT TCA ATT CTG AGG AGC TGG-3’</td>
</tr>
<tr>
<td>PKLR</td>
<td>5’ATC GTC TTT GCC TCC TTT GTG--3’</td>
<td>5’-TTT CAT CAA ACC TCT TCA CGC C-3’</td>
</tr>
</tbody>
</table>
Effects of interferon-gamma and energy metabolism pathways on intestinal epithelial gland morphogenesis in three-dimensional culture.