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Proteomic Profiling of Enteroid Cultures Skewed toward Development of Specific Epithelial Lineages

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Recently, 3D small intestinal organoids (enteroids) have been developed from cultures of intestinal stem cells which differentiate in vitro to generate all the differentiated epithelial cell types associated with the intestine and mimic the structural properties of the intestine observed in vivo. Small-molecule drug treatment can skew organoid epithelial cell differentiation toward particular lineages, and these skewed enteroids may provide useful tools to study specific epithelial cell populations, such as goblet and Paneth cells. However, the extent to which differentiated epithelial cell populations in these skewed enteroids represent their in vivo counterparts is not fully understood. This study utilises label-free quantitative proteomics to determine whether skewing murine enteroid cultures toward the goblet or Paneth cell lineages results in changes in abundance of proteins associated with these cell lineages in vivo. Here, proteomics data confirms that skewed enteroids recapitulate important features of the in vivo gut environment, demonstrating that they can serve as useful models for the investigation of normal and disease processes in the intestine. Furthermore, comparison of mass spectrometry data with histology data contained within the Human Protein Atlas identifies putative novel markers for goblet and Paneth cells.

The small intestine is organized into protruding finger-like villi, and crypts of Lieberkühn which extend into the muscularis mucosae. These structures are covered by a single layer of epithelium consisting of specialized cell types, including absorptive enterocytes, goblet cells, enteroendocrine cells, M cells, and Paneth cells.[1,2] Goblet and Paneth cells play crucial roles in protecting the host from microbial invasion, and in regulating the commensal flora. Goblet cells produce a protective mucus layer that is loosely adhered to the intestinal epithelium, and acts as a barrier to pathogen colonization and invasion.[3,4] Furthermore, they have been shown to play a role in luminal antigen sampling across the small intestinal epithelium.[5] Paneth cells reside at the crypt base, and secrete antimicrobial compounds into the crypt lumen following microbial stimulation.[6–9] Systems-based approaches have the potential to provide a more holistic view of the development and host-protective role of these epithelial cell populations. However, their relative scarcity in the epithelial cell layer, combined with complexities in the isolation and culture of these cells, pose a significant barrier to the application of unbiased profiling techniques, such as proteomics, to study these cells.

The small intestinal epithelium undergoes regular renewal via shedding of epithelial cells into the mucus layer, which is removed along with other gastrointestinal waste. Intestinal epithelial regeneration requires the presence of LGR5+ stem cells, which reside at the crypt base and are capable of generating all the specialized epithelial cell types found in the small intestine.[10,11] A Wnt gradient exits along the crypt-villus axis, originating in the crypt domain. Wnt-signaling maintains LGR5+ cell proliferation in the crypt. As daughter cells migrate along the Wnt gradient, crosstalk between Wnt, Notch, and BMP signaling determines cell fate, giving rise to differentiated epithelial lineages. These renewal properties can be exploited to generate 3D organoid cultures (called enteroids) from isolated intestinal LGR5+ stem cells or crypts.[12] When cultured in Matrigel® with a cocktail of growth factors, LGR5+ stem cells generate the differentiated epithelial cell types found in the small intestine, arranged in crypt-villus structures that mimic the complex intestinal architecture observed in vivo.[12] Since the initial description of these cultures in 2009, there has been a dramatic uptake in their use as in vitro models of a variety of different physiologic and pathologic processes. Consequently, characterization of the enteroid proteome would serve as a valuable resource in this growing field.

Enteroids can be treated with small molecule inhibitors to skew cell differentiation toward specific lineages.[13–15] For

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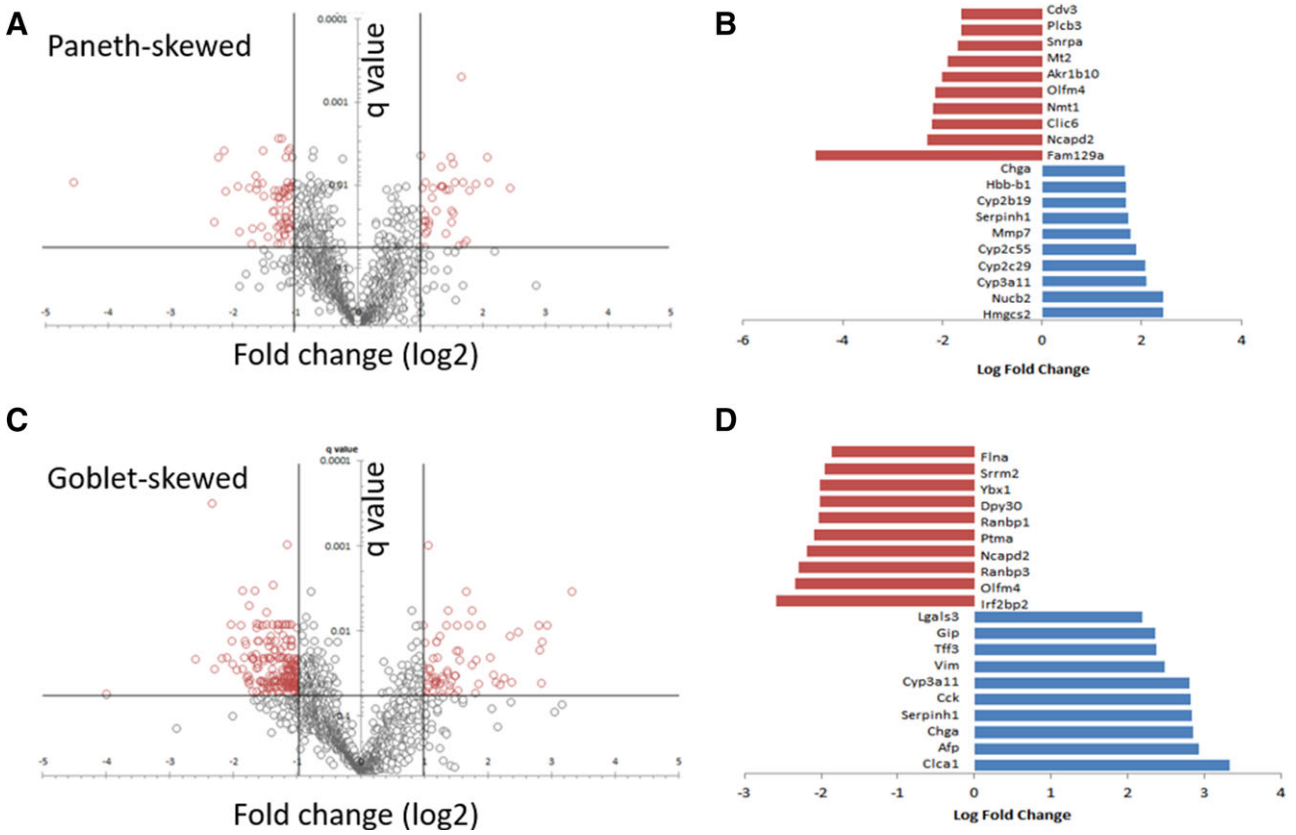

Figure 1. Drug treatment skews enteroids toward Paneth or goblet cell lineages. Intestinal enteroids were treated with DAPT/CHIR or DAPT/IWP-2 to promote differentiation toward the Paneth or goblet cell lineages, respectively. A) Volcano plot depicting changes in protein expression between control and Paneth-skewed enteroids. B) Top ten proteins up- and downregulated in Paneth-skewed enteroids C) Volcano plot depicting changes in protein expression between control and goblet-skewed enteroids. D) Top ten proteins up- and downregulated in goblet-skewed enteroids.

example, the combination of DAPT and CHIR99021, which inhibit notch signaling and GSK3β-mediated β-catenin degradation, respectively, directs epithelial cell differentiation toward the Paneth cell lineage.[15] If DAPT is instead combined with an inhibitor of Wnt signaling, IWP-2, epithelial cells are directed along the secretory cell lineages resulting in cultures enriched for goblet cells.[15] These skewed enteroids might allow systems approaches to be applied to study the host-defensive properties of specialized intestinal epithelial cells that have up until now evaded culture "in vitro." However, the extent to which differentiated epithelial cell populations generated from enteroids represent their in vivo counterparts is not fully understood.[16–23]

Four biological replicates of drug-skewed enteroid cultures were generated from murine small intestinal crypts essentially as previously described.[12] For drug skewing, media was changed on days two, five and seven to include 10 μM DAPT and either 3 μM CHIR99021 or 2 μM IWP-2 (Tocris, Oxford, UK).[15] On day eight, enteroids were fixed and prepared for confocal imaging as described in Supporting Information.

In DAPT and CHIR-treated enteroids ("Paneth-skewed"), we observed a higher proportion of cells stained with a Paneth cell marker, lysozyme, compared to control cultures (Figure S1A, Supporting Information). In DAPT and IWP-2-treated enteroids ("goblet-skewed"), we observed a greater proportion of cells stained with the goblet cell marker, MUC2, when compared to control enteroid cultures (Figure S1B, Supporting Information). These changes in expression of canonical markers of differentiated epithelial cell types confirm the success of small molecule inhibitors in directing the differentiation of LRG5+ stem cells toward specific epithelial cell lineages.

For mass spectrometry, a minimum of 50 organoids per treatment group were extracted from Matrigel using Cell Recovery Solution (BD Bioscience). Proteins were extracted in solution using 50 mM ammonium bicarbonate, 0.2% w/v Rapigest (Waters) and protein content was normalized between samples. Trypsin digested peptide mixtures (2 μL) were analyzed by online nanoflow liquid chromatography using the nanoACQUITY-nLC system (Waters MS technologies, Manchester, UK) coupled to an LTQ-Orbitrap Velos (ThermoFisher Scientific, Bremen, Germany) mass spectrometer with the manufacturer's nanospray ion source. Sample injections were not grouped by treatment type to avoid any batch bias. Protein identification and quantification were performed using Progenesis LC-MS for proteomics (v 4.1, Nonlinear Dynamics) and the Mascot search engine (v 2.3.02, Matrix Science), using the parameters described in Supporting Information. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier 10.6019/PXD005006.[2,3]

Using an exclusion criteria of ≥2 peptides identified, we identified a total of 1574 proteins in Paneth-skewed, and 1471

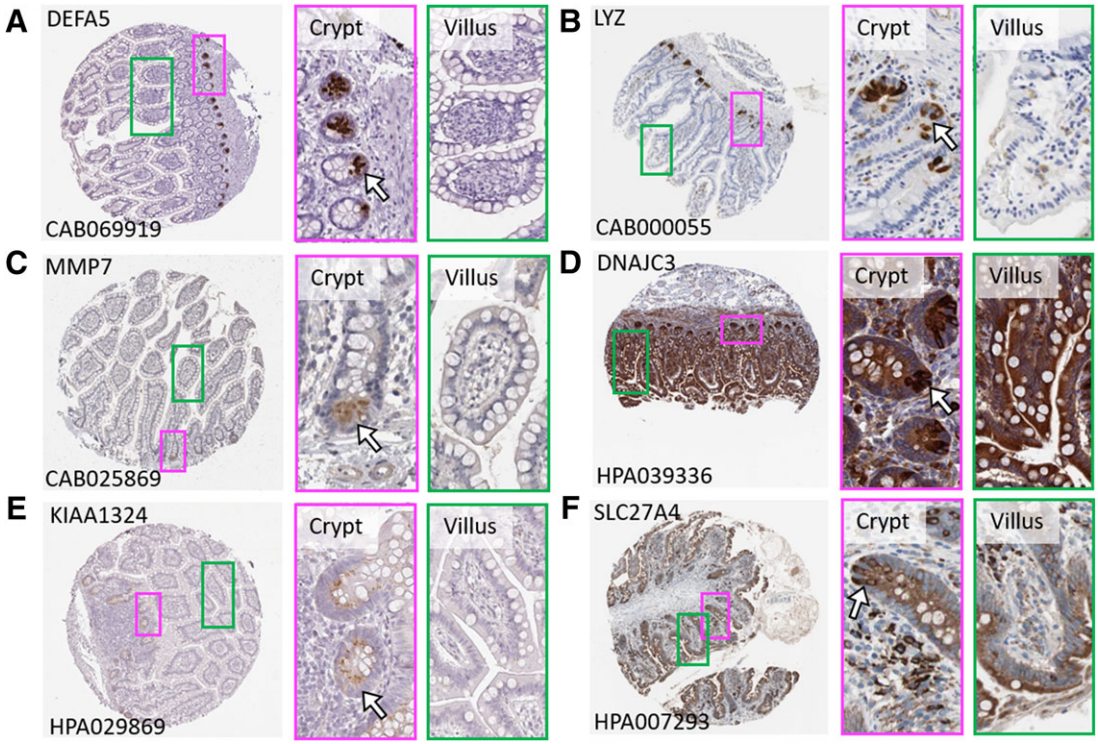

Figure 2. Intestinal expression patterns of proteins upregulated in Paneth-skewed cultures. Proteins upregulated in Paneth-skewed enteroids were cross-referenced with immuno-histochemical staining of normal human tissue available in the Protein Atlas. A–B) Immuno-histochemical staining for known Paneth cell markers DEFA5 and LYZ. C–G) Immuno-histochemical staining for a selection of proteins found to be upregulated in Paneth-skewed enteroid cultures, and also expressed in Paneth cells in human intestinal tissue. Pink regions highlight crypts, while green regions highlight villi. Arrows indicate Paneth cells (in crypt regions) or goblet cells (in villus regions). Images from v13.proteinatlas.org.

proteins in goblet-skewed enteroids. Applying exclusion criteria of $\log_2$ fold change >1 and q-value (ANOVA) < 0.05 (FDR adjusted p-value), 36 proteins were upregulated and 65 proteins were downregulated in Paneth-skewed enteroids compared to untreated controls (Figure 1A,B and Table S1, Supporting Information). In goblet-skewed enteroids, 55 proteins were upregulated, and 153 downregulated compared to untreated controls (Figure 1C,D and Table S2, Supporting Information). Thus, treatment with DAPT/CHIR or DAPT/IWP-2 results in distinct patterns of protein expression.

We next determined if the observed changes in protein abundance in Paneth-skewed cultures were reflective of known features of Paneth cells observed in vivo. Matrilysin (MMP7), a known marker of Paneth cells required for activation of pro-α-defensins, was significantly upregulated ($\log_2$ fold change = 1.78, q-value 0= 0.00093, Table S1, Supporting Information).[24] We also observed an increased abundance of several α-defensins (DEFA4, DEFA5, DEFA7, DEFA20, DEFA22, DEFA24) though none reached statistical significance. To confirm that proteins found to be significantly upregulated in Paneth-skewed cultures were also expressed by Paneth cells in vivo, we performed searches for human homologues of the proteins on the Human Protein Atlas (http://v13.proteinatlas.org, and Supporting Information).[25] Paneth cells were identified as granular cells residing at the base of small intestinal crypts, and antibody staining for Paneth cell products, lysozyme (LYZ), and defensin α5 (DEFA5), used as a reference (Figure 2A,B). Of the 36 proteins upregulated, expression of MMP7, KIAA1324, SLC27A4, and DNAJC3 was restricted to, or enriched within, Paneth-like cells (Figure 2C–F). Of these proteins, only MMP7 and DNAJC3 were uniquely upregulated in Paneth-skewed enteroids. To cope with their secretory demands, Paneth cells require a highly developed endoplasmic reticulum (ER)[26] which is protected from ER stress by the unfolded protein response (UPR). Intestinal epithelial cell-specific deletion of the UPR gene, *Xbp1*, leads to induction of ER stress, and a profound defect in Paneth cells.[27] Since DNAJC3 also plays a role in attenuation of ER stress,[26] we hypothesize that DNAJC3 may be important for Paneth cell development and function.

Goblet cells secrete mucins which form a protective mucus layer that maintains physical separation between the host epithelium and colonizing microbes. To determine if the observed changes in protein abundance in goblet-skewed cultures were reflective of differentiation toward the goblet cell lineage, proteomic profiles were cross-compared with a published database of murine small intestinal mucus components.[28] Of the 56 significantly upregulated proteins within our goblet-skewed enteroids, 14 (25%) were also detected in murine gastrointestinal mucus[28] (Table S3, Supporting Information). Of these, CLCA1 (log2 fold change = 3.328, q-value = 0.00347), AGR2 (log2 fold change = 1.857, q-value = 0.04257) and ZG16 (log2 fold change = 2.26, q-value = 0.03612) are among the most highly abundant constituents of gastrointestinal mucus. Our data therefore support the idea that goblet-skewed enteroid cultures

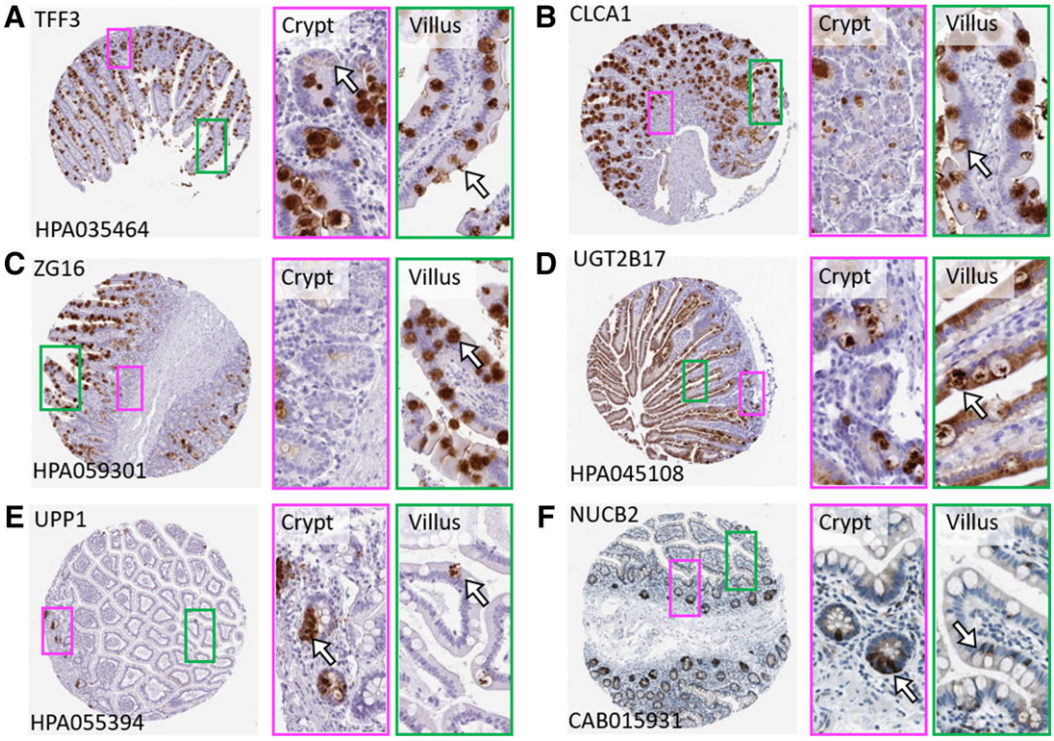

Figure 3. Intestinal expression patterns of proteins upregulated in goblet-skewed cultures. Proteins upregulated in goblet-skewed enteroids were cross-referenced with immuno-histochemical staining of normal human tissue available in the Protein Atlas. A) Immuno-histochemical staining for a known goblet cell marker, TFF3. A–D) Immuno-histochemical staining for a selection of proteins found to be upregulated in goblet-skewed enteroid cultures, and also expressed in goblet cells in human intestinal tissue. Pink regions highlight crypts, while green regions highlight villi. Arrows indicate Paneth cells (in crypt regions) or goblet cells (in villus regions). Images from v13.proteinatlas.org.

accurately recapitulate the in vivo environment, and may be useful models of goblet cell function. Indeed, goblet skewed colonic enteroids have been used to study the role of autophagy genes in mucus secretion.[29]

To further confirm that proteins found to be significantly upregulated in goblet-skewed cultures were also expressed by goblet cells in vivo, we again performed searches for human homologues of the proteins in The Human Protein Atlas (http://v13.proteinatlas.org, and Supporting Information).[25] Goblet cells were identified based on the presence of mucin granulae filling the cytoplasm at the apical surface, and antibody staining for a canonical marker of goblet cells, TFF3, was used as a reference (Figure 3A). Of the 55 proteins upregulated, TFF3, CLCA1, ZG16, and UGT2B17 expression was restricted to, or enriched within, goblet cells (Figure 3A–D). TFF3, CLCA1, and ZG16 have previously been associated with mucus production. Across a large panel of normal tissues encompassing all major organ systems, the Human Protein Atlas states that goblet cells show the strongest positivity for the remaining protein, UGT2B17.

Finally, we observed some commonalities in the proteins up- or downregulated in response to both DAPT/CHIR or DAPT/IWP-2 treatment. For example, OLFM4, an anti-apoptotic factor and marker of intestinal stem cells, was significantly downregulated under both treatment conditions, while expression of the canonical enteroendocrine cell marker, CHGA, was significantly upregulated in both Paneth- and goblet-skewed enteroids (Table S4, Supporting Information). This result is in close agreement with a previous study which used *chga* mRNA levels to show that treatment with both DAPT/CHIR and DAPT/IWP-2 results in increased differentiation toward the enteroendocrine lineage.[15] Finally, UPP1 and NUCB2 staining were observed in both Paneth and goblet cells (Figure 3E,F).

In this study, we have subjected murine enteroids to quantitative label-free proteomics, and shown that Paneth and goblet cells generated from intestinal stem cells in vitro share features typical of these cell types observed in vivo. This study has also led to the identification of novel protein markers not previously associated with these cell populations. Our data therefore support the use of Paneth- or goblet-skewed enteroids as a means of applying systems approaches to the study of infection of intestinal epithelial surfaces with pathogens.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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