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Molecular Profiling of Multiplexed Gene Markers to Assess Viability of Ex Vivo Human Colon Explant Cultures

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Abstract
Human colon tissue explant culture provides a physiologically relevant model system to study human gut biology. However, the small (20–30 mg) and complex tissue samples used present challenges for monitoring tissue stability, viability, and provision of sufficient tissue for analyses. Combining molecular profiling with explant culture has potential to overcome such limitations, permitting interrogation of complex gene regulation required to maintain gut mucosa in culture, monitor responses to culture environments and interventions. Human ex vivo colon explant gene expression profiles were assayed using an in-house custom-designed hCellMarkerPlex assay at culture time points 0, 1, 2, 4, and 14 h. Characteristic profiles of epithelial cell markers linked to differentiation, cellular polarization, and apoptosis were correlated with visible histochemical changes in explant epithelium during culture and tissue donors. The GenomeLab System provides effective assay of multiple targets not possible from small tissue samples with conventional gene expression technology platforms. This is advantageous to increase the utility of the ex vivo human colon model in applications to interrogate this complex and dynamic tissue environment for use in analytical testing.

Key words: cell marker; gut explant; microanatomy; molecular profile; multiplex

Introduction
Colon cell monocultures used extensively as an in vitro model system of the human gut provide limited information, failing to provide physiologically relevant data, or insights into the complex interactions between different cell types that comprise the colon mucosa.1 Colon cell lines are typically transformed, lack characteristic cellular architecture, exhibit different gene expression profiles in comparison with normal and tumor cells in the human body.1,2 Ex vivo cultured human colon tissue presents an alternative model system that is physiologically relevant to study human biology to generate data on metabolic responses and signaling pathways.3,4 Cultured explants consist of mucosa, a single layer of epithelial cells, the lamina propria and the muscularis mucosae.5,6 The epithelium consists of columnar epithelial cells (colonocytes), mucus-producing goblet cells, and scattered enteroendocrine cells, which form thin tubular glands known as crypts.5,6 The epithelial layer is perpetually renewed as a consequence of regulated proliferation of stem cells at the base of each crypt. The epithelium overlies the lamina propria, a cell-rich connective tissue containing fibroblasts, macrophages, lymphocytes, eosinophilic leukocytes, mast cells, and blood vessels.6 The epithelium and lamina propria are surrounded by a continuous sheet of smooth muscle, the muscularis mucosae.6 These small tissue
explants (typically 20–30 mg) limit comprehensive molecular analysis by conventional technology platforms. Monitoring donor variation and establishing normal as distinct from dysplastic tissue is important since tissue specimens are often obtained from patients attending for colectomy as a treatment for benign polyps or colorectal adenocarcinoma. This report investigates the application of in-house custom-designed gene expression assays\(^8,9\) to establish normal molecular profiles of human colon tissue explants, identify donor characteristics, and monitor cellular processes and aspects of tissue stability and viability within colon explants during culture.

**Materials and Methods**

**Human colon tissue**

Colectomy tissue was obtained through the Tayside Tissue Bank (Dundee, Scotland) from patients attending for colectomy as a treatment for benign polyps or colorectal adenocarcinoma (Ninewells Hospital, Dundee, Scotland). All patients consented for research use of tissues using the forms approved by the Tayside Local Research Ethics Committee through the Tayside Tissue Bank. Following visual assessment by a qualified pathologist, a piece of normal colon tissue comprising all tissue layers (mucosa, submucosa, muscle, subserosa, and serosa) was removed and placed in physiological saline solution (PSS; 119 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO\(_4\), 2.5 mM CaCl\(_2\), 11.1 mM glucose, pH = 7.6, 4°C) before preparation explants.

**Explant preparation and culture**

Mucosa was dissected in PSS at 4°C and explants (\(n = 14\)) prepared (3 × 3 mm in duplicate) using a scalpel. Explants were placed, mucosal layer uppermost, on wire mesh grids in six-well culture plates (Nunc GmbH & Co. KG) with \(\sim 3.5\) mL of culture media, RPMI (Sigma-Aldrich), and 1% fetal bovine serum [Cambrex (UK &Eire) Corp] to a level barely covering the explant. Culture plates were placed in a modular incubator chamber (MIC) (Billups-Rothenberg, Inc.) that was sealed and gassed with 95% O\(_2\)/5% CO\(_2\) for 10 min. Culture dishes in the MIC were continuously rocked (Grant BFR25 rocker; Cambridge Ltd) inside a Forma Scientific CO\(_2\) incubator (Forma Scientific UK Ltd) at 37°C. At culture time points 0, 1, 2, 4, and 14 h, duplicate explants were frozen in dry ice and stored at \(-80°C\) until use. The MIC was flushed with 95% O\(_2\)/5% CO\(_2\) for 10 min at each culture time point.

**Histological analysis**

Cryostat tissue sections (10 μm) were Hematoxylin and eosin (H&E) stained and viewed using a Leica DMR microscope [Leica Microsystems (UK) Ltd] and imaged using a QImaging QICAM Fast 1394 Digital CCD Camera (QImaging) and QCapture Pro 6.0 Software (QImaging).

**hCellMarkerPlex profiling of human colon explants**

Colon explant total RNA (50 ng in triplicate) samples (0, 1, 2, 4, and 14 h culture) were extracted and assayed using the hCellMarkerPlex and the GenomLab GeXP Start Kit (Beckman Coulter) as described previously.\(^8,9\) Yields were typically \(\sim 20\) μg with RIN values from 8 to 10.

**Statistical analysis**

Principal component analysis (PCA) was performed using SIMCA-P +12.0 software (MKS Instruments UK Ltd) on normalized hCellMarkerPlex assay data from colon explants (\(n = 14\) tissue donors) to assess expression patterns at culture time points tested and differences between tissue donor samples. Analysis of variance (ANOVA) was performed on normalized gene expression data, blocked for tissue donor with time points as treatment, using GenStat\(^\circ\) 13th Edition (VSN International, Ltd.). A *post hoc* Bonferroni correction for multiple comparisons of time points within an ANOVA was applied (significance level 0.05). ANOVA was conducted on a log scale if data were skewed.

**Results and Discussion**

Microanatomical analysis revealed normal histological epithelium and crypt structure that was maintained between 0 and 4 h culture time points (Fig. 1A–D). At 14 h, lamina propria, muscularis mucosae, and surface epithelium were still clearly visible, but loss of cell density in the lamina propria and reduced epithelial cell volume were observed (Fig. 1E). This was supported by the PCA biplot of normalized hCellMarkerPlex gene expression data (Fig. 1F). Colon explant profiles exhibited a gene expression pattern characteristic of normal tissue when compared to data from a previous study of normal colon, adenomatous polyp, and carcinoma tissues\(^8\) (Fig. 1F). Higher expression levels of epithelial markers MS4A12, EZR, and differentiation marker B4GANLT2, and lower expression levels of stem cell marker LGR5, proliferation markers PCNA and CCND1, and fibroblast marker COL1A1 (Fig. 1F)
FIG. 1.  Histological features of normal colon tissue in explant culture at 0 h (A), 1 h (B), 2 h (C), 4 h (D), and 14 h (E). Frozen tissue is Hematoxylin and eosin stained. Scale bar = 100 μm. (F) Biplot of the first two principle components (PCA plot) of hCellMarkerPlex gene expression data. The hCellMarkerPlex was applied to total RNA from colon explants cultured at 0 h (E0), 1 h (E1), 2 h (E2), 4 h (E4), and 14 h (E14) and compared with hCellMarkerPlex data from a previous study of human colon biopsy tissues, normal (N), adenomatous polyp (P), and carcinoma (T). Data have been normalized to UBE2D2. The PCA plot reveals clustering of colon explants and normal tissue indicating a greater similarity in gene expression profiles compared with the greater divergence in gene expression in adenomatous polyp and carcinoma. Gene targets are identified by name and location designated by ▲. The position of the gene targets signifies levels of expression characterizing the tissue types. e, epithelium; lp, lamina propria; m, muscularis mucosa; PCA, principal component analysis.
are associated with cultured explants and characterize normal tissue as opposed to colon adenomatous polyp or carcinoma tissues (Fig. 1F).

Microanatomical changes at 14 h were associated with increased expression of EZR, HDAC1, KRT18, B2M (a component of the major histocompatibility complex class I molecules), and CASP3 (an apoptotic marker). Elevated EZR may be a response to restore loss of epithelial stability as EZR is required to maintain a stable normal colon epithelium. Increased HDAC1 implies changes in gene regulation within the explant during culture. Elevated CASP3 may indicate the induction of apoptosis. Four of the gene targets displaying the highest degree of expression changes with time in culture, KRT18, SLC9A2, EZR, and ACTG2 are implicated in key roles in epithelial cell polarization. Little is known about epithelial cell polarization and complex cell–cell interactions within the human colon since it is difficult to recapitulate the dynamic transcriptional program of complex tissues using cell monocultures in vitro. Custom multiplexes can be designed to facilitate assay of small tissue specimens that specifically target components of signaling pathways to more fully capture this information.

FIG. 2. (A) Relative gene expression levels in human colon explants (n = 14) generated using the hCellMarkerPlex assay. The hCellMarkerPlex assay was applied to assess gene expression profiles of colon explant total RNA (50 ng in triplicate) samples extracted from cultured colon explants at 0, 1, 2, 4, and 14 h. The percentage of CV for each gene was calculated. The percentage of CV of 10% or less was achieved consistently for 20 of the genes within the hCellMarkerPlex (ACTG2, VWF, EZR, NOX1, HDAC1, UBE2D2, CCND1, B4GALNT2, SLC9A2, DES, LGR5, COL1A1, PCNA, CDX1, MS4A12, KRT18, FSP1, B2M, CDX2, and CASP3) as well as for the internal reference marker Kan(r). Low expressers (CNN1, MUC2, and NTN1) exhibited more variable percentage of CV ranging from 10% to 25%. GeNorm (http://medgen.ugent.be/genorm/) identified UBE2D2 as a consistently stable reference gene and data were thus normalized to UBE2D2. ANOVA blocked for tissue donor, with time points as treatment factors, was applied. A post hoc Bonferroni correction was applied and significant differences in the expression of each gene target between the culture time points tested is indicated by unique letters above each bar (p < 0.05). (B) B4GALNT2 expression in colon explants dissected from right and left colon. CV, coefficient of variation.
NOXI, B4GALNT2, SLC9A2, COL1A1, FSP1, and VWF were reduced at 14 h (Fig. 2A). NOXI and SLC9A2 regulate cellular pH and sodium transport across the apical membrane.17–19 Decreased B4GALNT2 (Fig. 2), a colon epithelial goblet cell differentiation marker,20 coincides with loss of epithelial cell volume. B4GALNT2 expression levels correspond with reports of a proximal-distal gradient of expression,20,21 with B4GALNT2 expression lower in the sigmoid and rectum compared to the cecum and ascending colon (Fig 2B). However, it was apparent that decreased B4GALNT2 expression was observed in all 14 h explants regardless of tissue location origin (Fig 2B). COL1A1 and FSP1, markers of fibroblasts within the lamina propria, produce collagen.22 Decreased COL1A1 and FSP1 were apparent throughout the culture (Fig. 2A), potentially a consequence of visible cell loss within the lamina propria.23 Expression of CNN1 (Fig. 2), a colon epithelial goblet cell differentiation marker,24 is linked to colon-specific epithelial marker that is specifically lost in explant culture (Fig. 2A). COL1A1 and FSP1 were ap-

parent throughout the culture (Fig. 2A), potentially a consequence of visible cell loss within the lamina propria (Fig. 1E). Donor variation in MUC2, NTN1, and CNN1 was largely attributed to low expression levels. Variation in CNN1 a smooth muscle marker,25,26 is likely a consequence of slight variation in smooth muscle attached to explants (Fig. 1A–E).

Eight gene markers associated with proliferation (PCNA, CCND1, MS4A12), differentiation (CDX1), apoptosis (NTN1), structural (CNN1, DES), and the stem cell marker LGR5, were not significantly altered during explant culture (Fig. 2A). MS4A12 is a CDX-regulated colon-specific epithelial marker that is specifically located in luminal surface epithelium25 and is linked to regulation of proliferation.26 CDX1, a member of the homeobox genes of the caudal family, is involved in epithelial differentiation.27 DES is a muscle cell marker.23 LGR5 is a colon stem cell marker, expressed within 4–6 cells at the base of each crypt,28 demonstrating the sensitivity of the hCellMarkerPlex. The lack of significant changes in the expression of these gene markers implies that there is no increased cell proliferation and differentiation on introduction of the explants to the culture system.

GeXP technology permits effective gene expression profiling in small tissue samples that is not possible using conventional technology platforms. The molecular profiles permit monitoring of tissue stability, viability, and donor characteristics. This is advantageous to increase utility of the ex vivo human colon model to interrogate this complex and dynamic tissue environment to generate physiologically relevant data on gene networks or signaling pathways and their roles within different cells and tissues. Development of further custom-designed assays will be a valuable tool for investigation of gene regulation in the very small tissue samples used for ex vivo colon explant culture to identify tissue responses to interventions.

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Author Disclosure Statement
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Abbreviations Used
ANOVA = analysis of variance
MIC = modular incubator chamber
PCA = Principal component analysis
PSS = physiological saline solution

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