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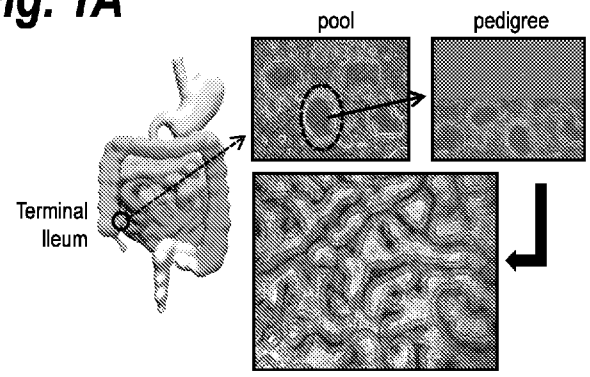
(54) Title: INFLAMMATORY BOWEL DISEASE STEM CELLS, AGENTS WHICH TARGET IBD STEM CELLS, AND USES RELATED THERETO

Fig. 1A



(57) Abstract: The present invention addresses IBD from the standpoint of mucosal stem cells cloned from defined regions of the gastrointestinal tract. In the case of pediatric Crohn's disease, for example, isolation of those stem cells according to the methods of the present invention reveals a pattern of inflammatory gene expression in stem cells from the terminal ileum and colon that is epigenetically maintained despite months of continuous cultivation in the absence of immune or stromal cells, or of intestinal microbes. Superimposed on this distributed inflammatory phenotype is a differentiation defect that profoundly and specifically alters the mucosal barrier properties of the terminal ileum. The co-existence of diseased and normal stem cells within the same endoscopic biopsies of Crohn's disease patients implicates an epigenetically enforced heterogeneity among mucosal stem cells in the dynamics of this condition.

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INFLAMMATORY BOWEL DISEASE STEM CELLS, AGENTS WHICH TARGET IBD STEM CELLS, AND USES RELATED THERETO

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 62/501,878, filed May 5, 2017. The entire contents of this application are incorporated herein in their entirety.

BACKGROUND

Inflammatory bowel disease (IBD) is a common disease of the Western World. Symptoms include chronic intestinal inflammation, diarrhea, bloody stool, weight loss and bowel obstruction. With no obvious cure, surgery is a frequent outcome. Major IBD-subtypes, Ulcerative colitis and Crohn's disease, share similar demographic and epidemiological features with as much as 10% of the cases being clinically indistinguishable. However, key differences in tissue damage and prognosis suggests distinct underlying pathogenic processes. In UC, inflammatory infiltrates and tissue damage is limited to the mucosal layer with extensive disruption of the mucosa, crypt abscesses, neutrophilic infiltrations. While transmural damage, thickening of intestinal wall and increased trichrome staining for connective tissue are typical of Crohn's disease.

IBD is classically viewed as a multi-step disease with two major players. First, initiating events of environmental origin, such as exotoxins, and other microbial factors. Secondly, the responding host immune system that leads to normal healing in unaffected, but inflammation and tissue response in IBD patients. Thus, past IBD studies have focused on selected environmental factors and cytokines, immune cells and inflammatory proteins.

Crohn’s disease is an inflammatory bowel disorder marked by transmural lesions that frequently progress to strictures, fistulas, or perforations requiring repeated surgical intervention. While its onset is typically in the third and fourth decade, about 15 percent of cases arise in children who tend to have a severe phenotype characterized by extensive small and large bowel disease, frequent need for corticosteroids and immunosuppression, growth delay, and enhanced risk for colorectal cancer. Though immunosuppressants and anti-inflammatory biologics can slow the progression of Crohn’s disease, it is not clear that they have lessened the need of surgical intervention, an impasse that has fueled the search for therapeutic targets more proximal to the disease. This search is complicated by the large environmental contribution to this disease reflected by the low concordance among monozygotic twins, and by the polygenic nature of the remaining, inherited risk. Nevertheless, genome-wide association (GWAS) and biological studies are beginning to define the underlying genetic structure and pathophysiology of Crohn’s disease. In

particular, there is a stunning overlap of risk loci associated with Crohn's and the susceptibility to mycobacterial infections, and many of the 170 risk loci discovered to date implicate genes of adaptive and innate immune processes that are likely involved in the containment of gut microbes. Consistent with this emerging "barrier defect" hypothesis are observations of profound deficiencies in the production of anti-microbial peptides by Paneth cells in Crohn's disease patients, defective autophagy processing of microbial antigens by mucosal epithelial cells and altered responsiveness of mucosal immune cells.

Despite its utility in characterizing the disease, the barrier defect hypothesis is mute on key features of the natural history of Crohn's disease including its alternate presentations as ileal, ileo-colonic, and colonic disease, the intermittent or "skip-lesion" patterning of inflammatory foci within these affected regions, and its cycle of flares and remission. Nor does this hypothesis address why the terminal ileum sustains the most severe manifestations of the disease necessitating surgery, or the nature and origin of recurrent disease that so frequently ensues the removal of the terminal ileum.

In the United States alone, more than 600,000 are affected every year. There is currently no satisfactory treatment, as the cause for IBD remains unclear although infectious and immunologic mechanisms have been proposed. IBD treatments aim at controlling inflammatory symptoms, conventionally using corticosteroids, aminosalicylates and standard immunosuppressive agents such as azathioprine (6-mercaptopurine), methotrexate and ciclosporine. Of these, the only disease-modifying therapies are the immunosuppressive agents azathioprine and methotrexate, both of which have a slow onset of action and only a moderate efficacy. Long-term therapy may cause liver damage (fibrosis or cirrhosis) and bone marrow suppression. Also patients often become refractory to such treatment. Other therapeutic regimes merely address symptoms.

SUMMARY

One aspect of the present invention provides isolated epithelial stem cells derived from gastrointestinal biopsies from IBD patients, referred to herein as "IBD Stem Cells". Prior to the invention, intestinal stem cells from disease samples were generally considered unviable in culture and isolation of stem cells from IBD patient biopsies and stable culture and passaging of those stem cells under conditions that maintain the genotype and epigenetics of the stem cell as it existed in the biopsy had not been described.

In certain embodiments, the subject IBD stem cells are derived from disease tissue samples such as biopsies from Crohn's Disease patients, including adult or pediatric patients. In other embodiments, the subject IBD stem cells are derived from disease tissue samples such as biopsies from Ulcerative Colitis patients, including adult or pediatric patients.

The isolation, passaging and maintenance of the subject IBD stem cells can be carried out using, for example, a culture media system comprising (a) a ROCK (Rho Kinase) inhibitor; (b) a Wnt agonist; (c); a Bone Morphogenetic Protein (BMP) antagonist; (d) a Notched Inhibitor; (e) a TGFβ signaling pathway inhibitor (e.g., a TGFβ inhibitor or a TGFβ receptor inhibitor), and (f) nicotinamide or an analog thereof. In another embodiment, the culture media system comprises (a) a ROCK (Rho Kinase) inhibitor; (b) a Wnt agonist; (c); a Bone Morphogenetic Protein (BMP) antagonist; (d) a Notched Inhibitor; (e) a TGFβ signaling pathway inhibitor (a TGFβ inhibitor or a TGFβ receptor inhibitor), and (f) nicotinamide or an analog thereof In certain embodiments, the the culture medium optionally further comprising a mitogenic growth factor and/or insulin or IGF. In certain embodiments, the cells from the IBD tissue sample, or the repassaged IBD stem cells, are optionally in fluid or direct contact with mitotically inactive feeder cells and/or in contact with extracellular matrix (such as a basement membrane matrix) or other bio- or synthetic matrix. In the case of the isolation of IBD stem cells from tissue samples, such as biopsies, the method can be performed by culturing dissociated epithelial cells from an IBD tissue sample in the medium, isolating single cells from the epithelial cell clones that arise, and culturing the isolated single cells from to form individual cultures of single cell clones, i.e., in contact with feeder cells and/or a basement membrane matrix in the medium, where each of the single cell clones represents a clonal expansion of the IBD epithelial stem cell.

Another aspect of the invention relates to the genes (“IBD gene sequences”) which are over- or under-expressed in IBD Stem cells, such as those genes which are differentiatally expressed relative to GI stem stem cells from patient matched normal tissue. These include gene sequences, such as the coding sequence, mRNA sequence, RNA transcipt or genonmic sequence for Atonal BHLH transcription factor 1 (ATOH1), MUC2, glycoprotein A33 (GPA33), claudin 18 (CLDN18), V-set and immunoglobulin domain containing 1 (VSIG1) or to the genes/proteins identified in Table 3, Figure 1D, Figure 19 are collectively herein the "pCD Gene Sequences". Genes which are upregulated or downregulated in IBD cells may be targets for diagnostic or therapeutic techniques.

The present invention makes available isolated polypeptides which are isolated from, or otherwise substantially free of other cellular proteins, especially other signal transduction factors and/or transcription factors which may normally be associated with the polypeptide. Subject polypeptides of the present invention include polypeptides encoded by pCD Gene Sequences. Polypeptides of the present invention include those proteins which are differentially regulated in IBD tissue, especially colon UC- and CD-derived cell lines (relative to normal cells, e.g., normal colon tissue).

In certain embodiments, the subject invention also provides antibodies which selectively bind to a polypetide gene expression product of an IBD gene sequences, such as

the pCD Gene Sequences, or other proteins upregulated in a population of IBD stem cells or its progeny, preferably a protein expressed on the cell surface of the IBD stem cell or its progeny. The antibodies of the present invention can be used, to illustrate, for both diagnostic or therapeutic benefits.

In certain embodiments, the antibody is an antibody-drug conjugate, such as an antibody which selectively bind to a polypetide gene expression product of a pCD Gene Sequence or other protein that is upregulated in a population of IBD stem cells or its progeny, which antibody is conjugated to a drug that has a cytotoxic effect, cytostatic effect or epigenetic effect on the IBD stem cell and/or its progeny.

The subject invention further provides a method of determining whether a cell sample obtained from a subject possesses an abnormal amount of marker polypeptide which comprises (a) obtaining a cell sample from the subject, (b) quantitatively determining the amount of the marker polypeptide in the sample so obtained, and (c) comparing the amount of the marker polypeptide so determined with a known standard, so as to thereby determine whether the cell sample obtained from the subject possesses an abnormal amount of the marker polypeptide. Such marker polypeptides may be detected by immunohistochemical assays, dot-blot assays, ELISA and the like.

Another aspect invention relates in part to novel methods for identifying and/or classifying patients with inflammatory bowel diseases (IBD), particularly patients with Crohn's disease or ulcerative colitis. Gene expression profiling, for the first time, shows broad and fundamental differences in the pathogenic mechanism of UC and CD. The subject method is based on the findings that certain genes are differentially expressed in intestinal tissue of IBD patients compared with related normal cells, such as normal colon cells. That change can be used to thereby identify or classify IBD cells by the upregulation and/or downregulation of expression of particular genes, alterations in protein levels or modification, or changes at the genomic level (such as mutation, methylation, etc), e.g., an event which is implicated in the pathology of inflammatory bowel diseases. Unlike prior methods, the invention provides a means for identifying IBD patients, and IBD cells at an early stage of development, so that treatment can be determined for early intervention. As described below, certain IBDs are associated with higher risks of cancer, e.g., colon cancer. This allows early detection of potentially cancerous conditions, and treatment of those cancerous conditions prior to spread of the cancerous cells throughout the body, or prior to development of an irreversible cancerous condition.

Still another aspect of the present invention provides drug screening assays for identifying agents which can be used to treat or manage the effects of an inflammatory bowel disease or disorder, e.g., by counteracting the effects of the up- or down-regulation of one or more of the subject IBD genes, such as the pCD Gene Sequences. Such assays

include formats which detect agents that inhibit or potentiate expression (transcription or translation) of an IBD gene, formats which detect agents that inhibit or potentiate an activity of an IBD gene product (enzymatic activity, protein-protein interaction, protein-DNA interaction, etc), formats which detect agents that which alter the splicing of IBD gene transcripts, and formats which detect agents that which shorten or extend the half-life of an IBD gene product. For each of the assay embodiments set out above, the assay is preferably repeated for a variegated library of at least 100 different test compounds, though preferably libraries of at least 10^3, 10^5, 10^7, and 10^9 compunds are tested. The test compound can be, for example, peptides, carbohydrates, nucleic acids and other small organic molecules, and/or natural product extracts.

In yet another aspect, the invention provides pharmaceutical compositions including agents, e.g., which have been identied by the assays described herein, which alter the level of expression or splicing of one or more IBD genes, alter the activity or half-life of an IBD gene product, or which alter the post-translational modification of an IBD gene product.

Another aspect of the present invention relates to transgenic non-human animals having germline and/or somatic cells in which the biological activity of one or more of the IBD Genes Set, such as one or more of the pCD Gene Sequences, are altered by a chromosomally incorporated transgene. Such animals can be used as models for inflammatory bowel diseases or disorders, e.g., for understanding the pathology of disease and/or drug screening.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1F. Mucosal stem cells from pediatric Crohn's

Figure 1A. Schematic of endoscopic sampling of biopsies from the terminal ileum (TI) and right colon (RC) and images of the pools of single cell-derived epithelial colonies from the TI in 2-D culture subsequent generation of discrete pedigrees for analysis. We cloned and propagated mucosal stem cells from endoscopic biopsies of children newly diagnosed with Crohn's disease and age-matched controls were cloned and propagated according to the methods of the present application. 150-200 independent stem cell colonies derived per 1 cubic millimeter biopsies of the terminal ileum. Stem cells were maintained in culture for >8 weeks as either "pools" of clones or as subcloned "pedigrees" derived from single cells. Pedigrees consist of highly immature stem cells that can be differentiated to a 3-D intestinal mucosa by exposure to an air-liquid interface

Figure 1B. Principal component analysis of whole genome expression profiles from discrete pedigrees of terminal ileum of Crohn's (CD1, CD2, and CD3), normal functional control (FC1), and 22-week fetus (fetal TI) as indicated. Cluster Analysis

revealed a bimodal distribution of gene expression profiles with a Normal cluster dominated by control and fetal terminal ileum pedigrees, and a Crohn's cluster occupied by most of the stem cell pedigrees from Crohn's patients.

Figure 1C. Heat map of differential gene expression (>1.5-fold, p<0.05) between stem cells of *Normal* category (FC1, fetal TI, subset of CD3) and Crohn's category (CD1, CD2, subset of CD3). The Crohn's cluster differentially expressed approximately 800 genes (>1.5-fold, p<0.05) compared to the Control cluster. Filtering these genes against known inflammatory gene sets yielded nearly 200 that populate innate immune and antigen presentation pathways previously linked to Crohn's.

Figure 1D. IPA analysis of genes both differentially expressed in Crohn's terminal ileum stem cells and overlapping with *Organ Inflammation* genes segregated bottom to top into nuclear, cytoplasmic, membrane, and secreted sets.

Figure 1E. Venn diagram of IPA Inflammation, genes differentially expressed in stem cells of Crohn's terminal ileum (TI) and of right colon (RC).

Figure 1F. Histogram of extent and polarity of expression of three-way overlapping genes of Venn diagram in *e*. For stem cells of the Crohn's cluster, gene expression profiling of ALI-differentiated terminal ileum stem cells reveals an enrichment of genes associated with inflammatory pathways involving antigen presentation, innate immune responses, cytokine signaling

Figures 2A–2E. *In vitro* differentiated epithelia from Crohn's stem cells

Figure 2A. *2-D* colonies of single cell-derived stem cell pedigree (*left*) and phase contrast image differentiated epithelia produced from pedigree (*right*).

Figure 2B. Expression heatmap comparing differentiated normal (FC1) and Crohn's (CD1, CD2) terminal ileum.

Figure 2C. Principal component analysis of whole genome transcription profile of stem cells and differentiated epithelia from normal (FC1) and Crohn's (CD1, CD2). The Air-Liquid interface differentiation of stem cell pedigrees amplified the distinctions between the Normal and Crohn's clusters to with nearly 1,200 genes (>1.5-fold, p<0.05).

Figure 2D. Most significant inflammatory pathways represented by genes differentially expressed in terminal ileum epithelia of Crohn's (CD1, CD2, CD3) relative to controls. For stem cells of the Crohn's cluster, gene expression profiling of ALI-differentiated terminal ileum stem cells reveals an enrichment of genes associated with inflammatory pathways involving antigen presentation, innate immune responses, cytokine signaling.

Figure 2E. Overlap between genes differentially expressed (>2-fold, $p<0.05$) in both CD1 and CD2 and gene sets defined by replicated GWAS loci in Crohn's.

Figures 3A-3F. Secretory cell defect in *in vitro*-differentiated Crohn's stem cells. Homeotic transformation of Crohn's stem cells.

Figures 3A and 3B. Crohn's stem cells fail to generate goblet, Paneth, and endocrine cells, and yet express other antigens (CLDN18 and VSIG1) normally expressed in proximal gastrointestinal tract. In addition to an extensive inflammatory gene signature and defective maturation of secretory cells, Crohn's cluster epithelia are distinguished by the ectopic expression of a host of metabolic enzymes that had no obvious links to either inflammation or secretory cell differentiation. BUT these enzymes, which function in the hydrolysis and transport of lipids, carbohydrates, and proteins, are normally expressed in proximal portions of gastrointestinal tract 12-14 feet anterior to the terminal ileum. Histological comparison between normal and Crohn's *in vitro*-differentiated terminal ileum via hematoxylin-eosin staining, and immunofluorescence of antibodies to Muc2/Ecad, CHGA/Ki67, DefA6, and GPA33.

Figure 3C. Comparative expression heatmap of genes associated with gastrointestinal goblet cells (ZG16, CLCA1), Paneth cells (DEFA5, DEFA6), and endocrine cells (SST, GCG) in *in vitro*-differentiated terminal ileum stem cells. Mapped the gene expression profiles of the Crohn's cluster terminal ileum stem cells against those of each region of the gastrointestinal tract and their corresponding differentiated epithelia derived from a 22-week fetal demise case. Analysis yielded a set of 271 genes that were both differentially expressed between Crohn's and Normal epithelia and showed regional expression along the normal fetal gastrointestinal tract. Pathway analysis of these genes showed the most significant categories to be related to the metabolism and transport of nutrients.

Figures 3D and 3E. Histological comparison of *in vitro*-differentiated normal (left) and Crohn's right colon stem cells via *(from top)* hematoxylin-eosin staining, and Muc2/Ecad, CHGA/Ki67, DefA6, and GPA33 immunofluorescence.

Figure 3F. Comparative expression intensity heatmap of genes associated with gastrointestinal goblet, Paneth, and enteroendocrine cells and Crohn's in general in *in vitro*-differentiated normal and Crohn's right colon stem cells.

Figure 3G. Stem cell pedigree from patient CD2 that binned with the Normal cluster differentiated to 3-D epithelial with the typical manifest of goblet cells, Paneth cells, and enteroendocrine cells produced by terminal ileum stem cells of control patients.

Figures 4A-4F. Homeotic transformation of Crohn's terminal ileum

Figure 4A. Immunohistochemical staining patterns across the gastrointestinal tract of metabolic enzymes differentially expressed in Crohn's terminal (*via Human Protein Atlas*).

Figure 4B. *Left*, Venn diagram of CD1 and CD2 terminal ileum gene sets selected for both differential expression in Crohn's and along fetal gastrointestinal tract. *Right*, Pathway analysis of overlapping genes in Venn diagram. p*z, combined p-value, z-score via *Enrichr* analysis (Mount Sinai Sch. Med.). Pathway analysis of these genes showed the most significant categories to be related to the metabolism and transport of nutrients. Mapped the gene expression profiles of the Crohn's cluster terminal ileum stem cells against those of each region of the gastrointestinal tract and their corresponding differentiated epithelia derived from a 22-week fetal demise case. Analysis yielded a set of 271 genes that were both differentially expressed between Crohn's and Normal epithelia and showed regional expression along the normal fetal gastrointestinal tract

Figure 4C. Mapping of genes differentially expressed (>1.5-fold, p<0.05) in *in vitro*-generated Crohn's terminal ileum to those differentially expressed (>1.8-fold, p<0.05) along the fetal gastrointestinal tract including over-represented in Crohn's (left) and over-represented in normal terminal epithelia (*right*). A, gastric fundus, B, gastric body, C, antrum, D, duodenum, E, jejunum, F, ileum, G, right colon, H, transverse colon, I, left colon. Mapping the genes over-represented in the Crohn's cluster terminal ileum epithelia to discrete regions of the fetal gastrointestinal tract revealed a shift in their distribution to one centered around gastric, duodenum and jejunum epithelia, whereas those over-represented in control terminal ileum generally mapped throughout the colon. Suggest that the terminal ileum stem cells of the Crohn's cluster displayed a switch in gene expression reminiscent of those driven by "homeotic" mutations.

Figure 4D. Mapping of genes differentially expressed (>1.5-fold, p<0.05) in Crohn's terminal ileum stem cells to those differentially expressed (>1.8-fold, p<0.05) along the fetal gastrointestinal tract including over-represented in Crohn's (*left*) and over-represented in normal terminal ileum stem cells (*right*).

Mapping the genes over-represented in the Crohn's cluster terminal ileum epithelia to discrete regions of the fetal gastrointestinal tract revealed a shift in their distribution to one centered around gastric, duodenum and jejunum epithelia, whereas those over-represented in control terminal ileum generally mapped throughout the colon. Suggest that the terminal ileum stem cells of the Crohn's cluster displayed a switch in gene expression reminiscent of those driven by "homeotic" mutations.

Figure 4E. Heatmap of differential gene expression (>1.8-fold, p<0.05) along the fetal gastrointestinal tract of inflammatory genes overexpressed (*left*) and underexpressed (*right*) (1.5-fold, p<0.05) in Crohn's terminal ileum stem cells post-differentiation.

Figure 4F. *Top*, Transcription factors implicated in the generation of indicated secretory cells in the gastrointestinal tract. *Bottom*, Differential expression of indicated transcription factors along the fetal gastrointestinal tract.

Figures 5A-5L. ATOH1 in secretory cell differentiation of intestinal mucosa

Figure 5A. Box plot of ATOH1 gene expression intensity for multiple terminal ileum stem cell pedigrees of Crohn's cases (CD1, CD2, CD3) and controls (FC1 and fetal terminal ileum). The top whisker shows maximum, the boxed zone shows 1st quartile, median and 3rd quartile, while the lower whisker shows minimum with outliers indicated (see Methods section).

Figure 5B. Clusters of Muc2/GFP-positive goblet cells in retrovirally transduced CD2 terminal ileum stem cells following differentiation in ALI culture.

Figure 5C. Differential gene expression heatmap comparing epithelia derived from normal (FC1) and ATOH1-transduced Crohn's (CD1, CD2) stem cells.

Figure 5D. Immunofluorescence micrographs of Muc2 and GFP expressing terminal ileum epithelia derived from ATOH1/GFP transduced and sorted CD2 stem cells.

Figure 5E. Histological sections of differentiated CD2 terminal ileum stem cells (*top*) and differentiated ATOH1-transduced and sorted CD2 stem cells (bottom).

Figure 5F. Expression heatmap of indicated secretory cell genes in differentiated terminal ileum CD1, CD2, as well as CD1 and CD2 following ATOH1 transduction.

Figure 5G. Histogram of differentially expressed genes in ATOH1-transduced CD1 and CD2 compared to corresponding CD1 and CD2 following ALI differentiation.

Figure 5H. Mapping of genes differentially expressed (>1.5-fold, p<0.05) in *in vitro*-generated CD2 terminal ileum to those differentially expressed (>1.8-fold, p<0.05) along the fetal gastrointestinal tract including over-represented in Crohn's (left) and over-represented in normal terminal epithelia (*right*).

Figure 5I. Mapping of genes differentially expressed (>1.5-fold, p<0.05) in *in vitro*-generated terminal ileum from ATOH1-transduced CD2 stem cells to those differentially expressed (>1.8-fold, p<0.05) along the fetal gastrointestinal tract including over-represented in CD2-ATOH1 (left) and over-represented in normal terminal epithelia (*right*).

Figure 5J. Generation and of biallelic frameshift and early stop codon in ATOH1 coding sequence in FC1 terminal ileum stem cells using CRISPR-Cas9-mediated

editing. Guide RNA, PAM sequence, and site of cleavage and final insertion of single nucleotide are indicated.

Figure 5K. Expression heatmap of genes associated with secretory cell types comparing differentiated FC1 stem cells and FC1 cells lacking ATOH1 expression ($FC1^{ATOH1-/-}$).

Figure 5L. Histological sections of differentiated FC1 terminal ileum stem cells and differentiated $FC1^{ATOH1-/-}$ stem cells.

Figures 6A-6E. Epigenetically maintained heterogeneity in Crohn's stem cells

Figure 6A. *Left*, Rhodamine red stained stem cell clones from CD3 terminal ileum biopsy on a lawn of irradiated feeder cells. *Right*, Differential anti-CLDN18 antibody staining of colonies within the CD3 pool of colonies.

Figure 6B. Characterization of two CD3 terminal ileum stem cell pedigrees having "Crohn's" and "Normal" expression profiles using phase contrast microscopy and immunofluorescence with antibodies to E-cadherin (ECAD), SOX9, and CLDN18.

Figure 6C. *In vitro* differentiation patterns in two distinct CD3 stem cell pedigrees having Crohn's (CD3-4K) and Normal (CD3-4D19) gene expression profiles.

Figure 6D. Principal component analysis of whole genome expression profiles of multiple CD3 terminal ileum stem cells and differentiated counterparts.

Figure 6E. Gene expression heatmap of differentiated CD3 terminal ileum stem cells along with normal controls (FC1) and Crohn's cases (CD1 and CD2).

Figure 7. GSEA of Crohn's terminal ileum stem cells. Most significantly enriched inflammatory pathways from differentially expressed genes in Crohn's terminal ileum stem cells.

Figure 8. Differential gene expression along fetal gastrointestinal tract. Heatmap of differentially expressed genes along the human fetal gastrointestinal tract stem cells and ALI differentiated epithelia.

Figure 9. Mapping differential gene expression in CD2 to fetal GI tract. Heatmap of differentially expressed genes along the human fetal gastrointestinal tract stem cells and ALI differentiated epithelia selected from gene set differentially expressed in the respective stem cells and ALI differentiated epithelia of CD2 terminal ileum.

Figure 10. Differential expression of homeotic genes along fetal GI tract. Heatmap of differentially expressed homeotic genes in stem cells and ALI differentiated epithelia derived from multiple regions of the human fetal gastrointestinal tract.

Figure 11. Targeted ATOH1 disruption in normal terminal ileum stem cells. Schematic of CRISPR-Cas9-mediated targeting and disruptive repair of the ATOH1 locus in normal terminal ileum stem cells and sequencing profiles of the ATOH1 loci in normal and targeted

cells. The disrupted alleles in the targeted cells show the insertion of single nucleotides (G/T).

Figure 12. Further exemplars of endoscopic sampling of biopsies from the Terminal Ileum (TI) and Right Colon (RC).

Figure 13. Crohn's Cluster Stem Cells of the Terminal Ileum show consistent abnormalities in both secretory cell differentiation and intercellular junctional assembly. Altered expression patterns in CD terminal ileum epithelial are supported by a broader comparison of expression profiles of particular genes associated with goblet, Paneth, and endocrine cells, all of which were low relative to control terminal ileum. Among pathways apparently under-represented in the Crohn's epithelia were inflammasome signaling, LPS and IL-1 mediated inhibition of retinoid signaling, certain steroid receptor (LXR/RXR and FXR/RXR) signaling, and serotonin degradation. The simultaneous loss and gain of particular inflammatory pathways is consistent with emerging concepts of Crohn's as both a pro-inflammatory condition and one remarkable defective in managing the containment of intestinal microbes

Figure 14. Traits of inflammation, secretory cell defects, and homeotic transformations of terminal ileum stem cells are interdependent. Approximately 80% of the genes over-represented in the inflammatory signature are normally expressed in proximal (gastric, duodenum, jejunum) portions of the gastrointestinal tract but not in the colon. This represents a shift to producing epithelial linings of absorptive function and is an explanation for ongoing inflammatory signals initiating from the terminal ileum lining.

Figures 15A and 15B. Homeotic transformation of Crohn's stem cells. In addition to an extensive inflammatory gene signature and defective maturation of secretory cells, Crohn's cluster epithelia are distinguished by the ectopic expression of a host of metabolic enzymes that had no obvious links to either inflammation or secretory cell differentiation. Howefver these enzymes, which function in the hydrolysis and transport of lipids, carbohydrates, and proteins, are normally expressed in proximal portions of gastrointestinal tract 12-14 feet anterior to the terminal ileum.

Figures 16A, 16B and 16C. Homeotic transformation of Crohn's stem cells. To further illustrate, using whole-genome analyses of epigenetic histone marks, we identified multiple alterations in the epigenetic profiles of the HOX loci of stem cells of the Crohn's cluster compared to those of the Normal cluster. Among these genes are a host of transcription factors including CDX2 and GATA5, whose respective roles distal and proximal gastrointestinal tract differentiation are well established.

Figures 17A and 17B. Inflammatory signature driven by homeotic shift in Crohn's stem cells. Consistent with the stability of the inflammatory signature in the Crohn's stem cells, 71% of the 180 genes showing enhanced expression in the inflammatory signature also had differential histone modifications marked by increased H3K4-trimethylation and a loss of

H3K27-trimethylation typical of active or potentially active genes. Of down-regulated genes in the inflammatory gene signature, 48% had differential histone modifications and these were dominated by a loss of H3K27-acetylation and a gain of H3K27-trimethylation associated with gene repression. Examples of such over- and under-represented genes with histone modifications include the transcription factor aryl hydrocarbon receptor (AHR) and the enterotoxin receptor GUCY2C.

Figure 18. The molecular genetics of the nodal genes responsible for this identity shift can be identified using the system of the present application. Epigenetic repression of homeoboxtranscription factors typically expressed in distal gastrointestinal tract in Crohn's Cluster stem cells paralleling similar differences between normal colon and proximal intestine. Conversely, homeobox transcription factors upregulated in Crohn's Cluster stem cells show enhanced activating marks (H3K4me3) paralleling similar differences between normal colon and proximal gastrointestinal tract.

Figure 19A-19B. Inflammatory signature driven by homeotic shift.

Figure 19A. A sampling of genes whose over- (e.g. AHR, IL1RN, MGAT5, SMAD3, FUT2, and DUOX2) and under- (e.g. CLCA1, GUCY2C, IL37, and NOX1) representation might be a consequence of the homeotic transformation underscores the potential immunoregulatory impact of such a conversion.

Figure 19B. Comparing the relationship between Crohn's cluster stem cells and the genetic architecture of Crohn's Disease. Comparison of the set of genes linked by GWAS to Crohn's from multiple studies with those differentially expressed genes by Crohn's cluster stem cells and the epithelia generated from them and those predicted by disease association algorithms (e.g. GRAIL). Overlap of 28% of genes implicated by GRAIL and those differentially expressed by the Crohn's cluster cells, as well as another set of genes that were not captured by GRAIL and whose significance Tract is exploring. The overlap between genes implicated by GWAS and those differentially expressed in Crohn's disease epithelia suggests broad links between these mucosal stem cells and the disease process.

Figures 20 and 21. Crohn's stem cells and normal terminal ileum stem cells can be readily adapted to multiwell culture formats for high throughput drug screening. Screening with libraries of compounds of reveal the opportunity to develop selective cytotoxic agents and synergistic drug combinations.

Figure 22. Animal model generating ileum xenografts, with polarization and myofibroblasts. This xenograft model of the present invention entails a simple subcutaneous injection of stem cell pools or clones in Matrigel into immunodeficient mice. By two weeks, these cells form a polarized normal or Crohn's stem cells around an acellular lumen. Remarkably, the vesicles generated by Crohn's epithelia become surrounded by a dense mat of α-smooth

muscle actin (SMA)-positive myofibroblasts whereas the normal control ileum stem cells do not. In the immunodeficient mice at least, this fibrosis is not accompanied by macrophages or other obvious leukocytes, suggesting the trigger is not going through such cells but rather directly via the Crohn's stem cells.

Figure 23. B allele frequency (BAF) and log R ratio (LRR) plots show the genetic stability of a colon ground state stem cells clone from passage 0 to passage 25. This stability has been observed beyond passage 75.

DETAILED DESCRIPTION

I. Overview

Crohn's disease is an inflammatory bowel disorder marked by transmural lesions that frequently progress to strictures, fistulas, or perforations requiring repeated surgical intervention[1]. While its onset is typically in young adults, 15 percent of cases arise in children who tend to have severe and extensive disease, frequent need for corticosteroids and immunosuppression, and enhanced risk for colorectal cancer[2]. Though immunosuppressants and anti-inflammatory biologics can slow the progression of Crohn's disease, it is not clear that they have lessened the need of surgical intervention, an impasse that has fueled the search for therapeutic targets more proximal to the disease[3]. This search is complicated by the large environmental contribution to this disease reflected by the low concordance among monozygotic twins[4], and by the polygenic nature of the remaining, inherited risk. Nevertheless, genome-wide association studies (GWAS) and pathophysiology are beginning to define the underlying genetic structure and biology of Crohn's disease[5,6]. In particular, there is a stunning overlap of risk loci for Crohn's and mycobacterial infections[7], and many of the 170 loci discovered to date implicate genes of adaptive and innate immune processes that are likely involved in the containment of gut microbes[7-10]. Consistent with this emerging "barrier defect" hypothesis are deficiencies in antimicrobial functions of Paneth cells in Crohn's disease patients[11], defective autophagy processing of microbial antigens by mucosal epithelial cells[11-17] and altered responsiveness of mucosal immune cells[18]. Despite these emerging data on mucosal barrier abnormalities in Crohn's, it remains unclear whether they are primary events or secondary consequences of the inflammatory state of this disease. It is also unclear how defective barrier function might explain the alternate regional presentations of Crohn's[19], its skip-lesion patterning, or the high rates of recurrence following ileo-colonic resection[1,3].

Most approaches to the treatment of Crohn's disease, Ulcerative Colitis and other forms of Inflammatory Bowel Diseases (IBD) focus on reducing or inhibiting the inflammatory components of these diseases. However, as described here, the inflammatory symptoms of Crohn's and other forms of IBD are a consequence of an altered epithelial lining generated

by an epigenetically shifted stem cell in the tissue – with inflammation being caused by the altered epithelia. As described in greater detail below and the attached figures, in the case of inflammatory bowel diseases such as Crohn's, we have found an epigenetic shift in the stem cells of the terminal ileum – where the inflammatory storm that characterizes this disease occurs. In this case, the stem cells that give rise to the lining of the terminal ileum are altered in a way that cause them to give rise to an epithelial lining that is similar to what occurs further up the digestive tract where absorption of nutrients occur and is not serving as a barrier to bacteria the way the terminal ileum should. In addition, this epigenetic change to the stem cells is also turning on genes which attract immune cells, such as a signals and activators of the innate and/or adaptive immune systems. Whatever the initial insult is that causes this shift, the immune response in the gut is perpetuated by this altered epithelial lining produced by these stem cells.

The present invention addresses IBD from the standpoint of mucosal stem cells cloned from defined regions of the gastrointestinal tract. In the case of pediatric Crohn's disease, for example, isolation of those stem cells according to the methods of the present invention reveals a pattern of inflammatory gene expression in stem cells from the terminal ileum and colon that is epigenetically maintained despite months of continuous cultivation in the absence of immune or stromal cells, or of intestinal microbes. Superimposed on this distributed inflammatory phenotype is a differentiation defect that profoundly and specifically alters the mucosal barrier properties of the terminal ileum. And while the immediate basis of this barrier defect can be traced to a loss of ATOH1, a transcription factor required for secretory cell differentiation in the colon, this repression of ATOH1 is only emblematic of a more profound alteration of the terminal ileum in Crohn's disease involving a homeotic transformation of stem cells to a developmental ground state represented by the duodenum and jejunum. Lastly, the co-existence of diseased and normal stem cells within the same endoscopic biopsies of Crohn's disease patients implicates an epigenetically enforced heterogeneity among mucosal stem cells in the dynamics of this condition.

II. Definitions

"Inflammatory bowel disease", or "IBD", is a term that encompasses both ulcerative colitis (inflammation of the lining of the large intestine) and Crohn's disease (inflammation of the lining and wall of the large and/or small intestine). When inflamed, the lining of the intestinal wall is red and swollen, becomes ulcerated, and bleeds. Although lesions associated with IBD can heal by themselves, most are recurrent. Chronic lesions occur in individuals with underlying diseases of various types whose medical conditions compromise the body's ability to repair injured tissue on its own (e.g., diabetes).

One type of lesion associated with IBD is an ulcer. A lesion is an open sore, an abrasion, a blister, or a shallow crater resulting from the sloughing or erosion of the top layer of epithelial cells and, sometimes, subcutaneous tissues. Although an ulcer can technically occur anywhere on the skin (e.g., a wound), the term “ulcer”, which is used loosely and interchangeably with “gastric ulcer” and “peptic ulcer”, usually refers to disorders in the upper digestive tract.

The term “an aberrant expression”, as applied to a nucleic acid of the present invention, refers to level of expression of that nucleic acid which differs from the level of expression of that nucleic acid in healthy gastroinstestinal tissue, or which differs from the activity of the polypeptide present in a healthy subject. An activity of a polypeptide can be aberrant because it is stronger than the activity of its native counterpart. Alternatively, an activity can be aberrant because it is weaker or absent relative to the activity of its native counterpart. An aberrant activity can also be a change in the activity; for example, an aberrant polypeptide can interact with a different target peptide. A cell can have an aberrant expression level of a gene due to overexpression or underexpression of that gene.

“Amino acid sequence” as used herein refers to an oligopeptide, peptide, polypeptide, or protein sequence, and fragment thereof, and to naturally occurring or synthetic molecules. Fragments of an expression product of an IBD gene sequence (an “IBD gene product”) are preferably about 5 to about 15 amino acids in length and retain the biological activity or the immunological activity of an IBD gene product. Where “amino acid sequence” is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, amino acid sequence, and like terms, are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

The term "antibody" broadly refers to any immunoglobulin (Ig) molecule and immunologically active portions of immunoglobulin molecules (i.e., molecules that contain an antigen binding site that immunospecifically bind an antigen) comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains, or any functional fragment, mutant, variant, or derivation thereof, which retains the essential epitope binding features of an Ig molecule. Such mutant, variant, or derivative antibody formats are known in the art. Nonlimiting embodiments of which are discussed below, and include but are not limited to a variety of forms, including full length antibodies and antigen-binding portions thereof; including, for example, an immunoglobulin molecule, a monoclonal antibody, a chimeric antibody, a CDR-grafted antibody, a human antibody, a humanized antibody, a single chain antibody, a Fab, a F(ab'), a F(ab')2, a Fv antibody, fragments produced by a Fab expression library, a disulfide linked Fv, a scFv, a single domain antibody (dAb), a diabody, a multispecific antibody, a dual specific antibody, an anti-idiotypic antibody, a bispecific

antibody, a functionally active epitope-binding fragment thereof, bifunctional hybrid antibodies (e.g., Lanzavecchia et al., Eur. J. Immunol. 17, 105 (1987)) and single chains (e.g., Huston et al., Proc. Natl. Acad. Sci. U.S.A., 85, 5879-5883 (1988) and Bird et al., Science 242, 423-426 (1988), which are incorporated herein by reference) and/or antigen-binding fragments of any of the above (See, generally, Hood et al., Immunology, Benjamin, N.Y., 2ND ed. (1984), Harlow and Lane, Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory (1988) and Hunkapiller and Hood, Nature, 323, 15-16 (1986), which are incorporated herein by reference). Antibodies also refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain antigen or target binding sites or "antigen-binding fragments." The antibody or immunoglobulin molecules described herein can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule, as is understood by one of skill in the art. Furthermore, in humans, the light chain can be a kappa chain or a lambda chain.

The term “specific affinity binder” refers to an antibody as well as to a non-antibody protein scaffold i.e., smaller proteins that are capable of achieving comparable affinity and specificity using molecular structures that can be for example one-fifth to one-tenth the size of full antibodies, and also to nucleic acid aptamers. In some embodiments, the specific affinity binder of the present invention is a non-antibody polypeptide. In some embodiments, the non-antibody polypeptide can include but is not limited to peptibodies, DARPins, avimers, adnectins, anticalins, affibodies, affilins, atrimers, bicyclic peptides, centryins, Cys-knots, Fynomers, Kunitz domains, Obodies, pronectins, Tn3, maxibodies, or other protein structural scaffold, or a combination thereof.

A disease, disorder, or condition “associated with” or “characterized by” an aberrant expression of an IBD gene sequence refers to a disease, disorder, or condition in a subject which is caused by, contributed to by, or causative of an aberrant level of expression of a nucleic acid.

“Biological activity” or “bioactivity” or “activity” or “biological function”, which are used interchangeably, herein mean an effector or antigenic function that is directly or indirectly performed by a polypeptide (whether in its native or denatured conformation), or by any subsequence thereof. Biological activities include binding to polypeptides, binding to other proteins or molecules, activity as a DNA binding protein, as a transcription regulator, ability to bind damaged DNA, etc. A bioactivity can be modulated by directly affecting the subject polypeptide. Alternatively, a bioactivity can be altered by modulating the level of the polypeptide, such as by modulating expression of the corresponding gene.

The terms “complementary” or “complementarity,” as used herein, refer to the natural binding of polynucleotides under permissive salt and temperature conditions by base-pairing.

For example, the sequence “A-G-T” binds to the complementary sequence “T-C-A”. Complementarity between two single-stranded molecules may be “partial”, in which only some of the nucleic acids bind, or it may be complete when total complementarity exists between the single stranded molecules. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, which depend upon binding between nucleic acids strands and in the design and use of PNA molecules.

A “composition comprising a given polynucleotide sequence” as used herein refers broadly to any composition containing the given polynucleotide sequence. The composition may comprise a dry formulation or an aqueous solution. Compositions comprising polynucleotide sequences encoding an IBD gene product or fragments thereof may be employed as hybridization probes. The probes may be stored in freeze-dried form and may be associated with a stabilizing agent such as a carbohydrate. In hybridizations, the probe may be deployed in an aqueous solution containing salts (e.g., NaCl), detergents (e.g., SDS) and other components (e.g., Denhardt's solution, dry milk, salmon sperm DNA, etc.).

The term “correlates with expression of a polynucleotide”, as used herein, indicates that the detection of the presence of ribonucleic acid that is similar to one of IBD genes by northern analysis is indicative of the presence of mRNA encoding an IBD gene product in a sample and thereby correlates with expression of the transcript from the polynucleotide encoding the protein.

A “deletion”, as used herein, refers to a change in the amino acid or nucleotide sequence and results in the absence of one or more amino acid residues or nucleotides.

As is well known, genes or a particular polypeptide may exist in single or multiple copies within the genome of an individual. Such duplicate genes may be identical or may have certain modifications, including nucleotide substitutions, additions or deletions, which all still code for polypeptides having substantially the same activity. The term “DNA sequence encoding an IBD polypeptide” may thus refer to one or more genes within a particular individual. Moreover, certain differences in nucleotide sequences may exist between individual organisms, which are called alleles. Such allelic differences may or may not result in differences in amino acid sequence of the encoded polypeptide yet still encode a polypeptide with the same biological activity.

As used herein, the terms “gene”, “recombinant gene”, and “gene construct” refer to a nucleic acid of the present invention associated with an open reading frame, including both exon and (optionally) intron sequences.

A “recombinant gene” refers to nucleic acid encoding a polypeptide and comprising exon sequences, though it may optionally include intron sequences which are derived from, for example, a related or unrelated chromosomal gene. The term “intron” refers to a DNA

sequence present in a given gene which is not translated into protein and is generally found between exons.

The term “growth” or “growth state” of a cell refers to the proliferative state of a cell as well as to its differentiative state. Accordingly, the term refers to the phase of the cell cycle in which the cell is, e.g., G0, G1, G2, prophase, metaphase, or telophase, as well as to its state of differentiation, e.g., undifferentiated, partially differentiated, or fully differentiated. Without wanting to be limited, differentiation of a cell is usually accompanied by a decrease in the proliferative rate of a cell.

“Homology” or “identity” or “similarity” refers to sequence similarity between two peptides or between two nucleic acid molecules, with identity being a more strict comparison. Homology and identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are identical at that position. A degree of homology or similarity or identity between nucleic acid sequences is a function of the number of identical or matching nucleotides at positions shared by the nucleic acid sequences. A degree of identity of amino acid sequences is a function of the number of identical amino acids at positions shared by the amino acid sequences. A degree of homology or similarity of amino acid sequences is a function of the number of amino acids, i.e., structurally related, at positions shared by the amino acid sequences. An “unrelated” or “non-homologous” sequence shares less than 40% identity, though preferably less than 25% identity, with one of the sequences of the present invention.

The term “percent identical” refers to sequence identity between two amino acid sequences or between two nucleotide sequences. Identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same base or amino acid, then the molecules are identical at that position; when the equivalent site occupied by the same or a similar amino acid residue (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous (similar) at that position. Expression as a percentage of homology, similarity, or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. Various alignment algorithms and/or programs may be used, including FASTA, BLAST, or ENTREZ. FASTA and BLAST are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default settings. ENTREZ is available through the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md. In one embodiment, the percent identity of two sequences can be determined by the GCG program

with a gap weight of 1, e.g., each amino acid gap is weighted as if it were a single amino acid or nucleotide mismatch between the two sequences.

Other techniques for alignment are described in Methods in Enzymology, vol. 266: Computer Methods for Macromolecular Sequence Analysis (1996), ed. Doolittle, Academic Press, Inc., a division of Harcourt Brace & Co., San Diego, Calif., USA. Preferably, an alignment program that permits gaps in the sequence is utilized to align the sequences. The Smith-Waterman is one type of algorithm that permits gaps in sequence alignments. See Meth. Mol. Biol. 70: 173-187 (1997). Also, the GAP program using the Needleman and Wunsch alignment method can be utilized to align sequences. An alternative search strategy uses MPSRCH software, which runs on a MASPAR computer. MPSRCH uses a Smith-Waterman algorithm to score sequences on a massively parallel computer. This approach improves ability to pick up distantly related matches, and is especially tolerant of small gaps and nucleotide sequence errors. Nucleic acid-encoded amino acid sequences can be used to search both protein and DNA databases.

Databases with individual sequences are described in Methods in Enzymology, ed. Doolittle, supra. Databases include Genbank, EMBL, and DNA Database of Japan (DDBJ).

The term “hybridization”, as used herein, refers to any process by which a strand of nucleic acid binds with a complementary strand through base pairing.

An “insertion” or “addition”, as used herein, refers to a change in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid residues or nucleotides, respectively, as compared to the naturally occurring molecule.

The term “isolated” as used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs, or RNAs, respectively, that are present in the natural source of the macromolecule. The term isolated as used herein also refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an “isolated nucleic acid” is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state. The term “isolated” is also used herein to refer to polypeptides which are isolated from other cellular proteins and is meant to encompass both purified and recombinant polypeptides.

“Microarray” refers to an array of distinct polynucleotides or oligonucleotides synthesized on a substrate, such as paper, nylon or other type of membrane, filter, chip, glass slide, or any other suitable solid support.

The terms “modulated” and “differentially regulated” as used herein refer to both upregulation (i.e., activation or stimulation (e.g., by agonizing or potentiating)) and

downregulation (i.e., inhibition or suppression (e.g., by antagonizing, decreasing or inhibiting)).

The term “mutated gene” refers to an allelic form of a gene, which is capable of altering the phenotype of a subject having the mutated gene relative to a subject which does not have the mutated gene. If a subject must be homozygous for this mutation to have an altered phenotype, the mutation is said to be recessive. If one copy of the mutated gene is sufficient to alter the genotype of the subject, the mutation is said to be dominant. If a subject has one copy of the mutated gene and has a phenotype that is intermediate between that of a homozygous and that of a heterozygous subject (for that gene), the mutation is said to be co-dominant.

As used herein, the term “nucleic acid” refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides. ESTs, chromosomes, cDNAs, mRNAs, and rRNAs are representative examples of molecules that may be referred to as nucleic acids.

As used herein, "gene silencing" or "gene silenced" in reference to an activity of an RNAi molecule, for example a siRNA or miRNA refers to a decrease in the mRNA level in a cell for a target gene (i.e., an IBD gene sequence) by at least about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 99%, about 100% of the mRNA level found in the cell without the presence of the miRNA or RNA interference molecule. In one preferred embodiment, the mRNA levels are decreased by at least about 70%, about 80%, about 90%, about 95%, about 99%, about 100%.

As used herein, the term "RNAi" refers to any type of interfering RNA, including but not limited to, siRNAi, shRNAi, endogenous microRNA and artificial microRNA. For instance, it includes sequences previously identified as siRNA, regardless of the mechanism of downstream processing of the RNA (i.e. although siRNAs are believed to have a specific method of in vivo processing resulting in the cleavage of mRNA, such sequences can be incorporated into the vectors in the context of the flanking sequences described herein). The term "RNAi" can include both gene silencing RNAi molecules, and also RNAi effector molecules which activate the expression of a gene. By way of an example only, in some embodiments RNAi agents which serve to inhibit or gene silence are useful in the methods, kits and compositions disclosed herein to alter the expression of, such as in particular inhibit the expression of an IBD gene sequence.

As used herein, a "siRNA" refers to a nucleic acid that forms a double stranded RNA, which double stranded RNA has the ability to reduce or inhibit expression of a target IBD gene sequence when the siRNA is present or expressed in the same cell as the target gene. The double stranded RNA siRNA can be formed by the complementary strands. In one embodiment, a siRNA refers to a nucleic acid that can form a double stranded siRNA. The sequence of the siRNA can correspond to the full-length target gene, or a subsequence thereof. Typically, the siRNA is at least about 15-50 nucleotides in length (e.g., each complementary sequence of the double stranded siRNA is about 15-50 nucleotides in length, and the double stranded siRNA is about 15-50 base pairs in length, preferably about 19-30 base nucleotides, preferably about 20-25 nucleotides in length, e.g., 20, 21 , 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length).

As used herein "shRNA" or "small hairpin RNA" (also called stem loop) is a type of siRNA. In one embodiment, these shRNAs are composed of a short, e.g. about 19 to about 25 nucleotide, antisense strand, followed by a nucleotide loop of about 5 to about 9 nucleotides, and the analogous sense strand. Alternatively, the sense strand can precede the nucleotide loop structure and the antisense strand can follow.

The terms "microRNA" or "miRNA" are used interchangeably herein are endogenous RNAs, some of which are known to regulate the expression of protein-coding genes at the posttranscriptional level. Endogenous microRNAs are small RNAs naturally present in the genome that are capable of modulating the productive utilization of mRNA. The term artificial microRNA includes any type of RNA sequence, other than endogenous microRNA, which is capable of modulating the productive utilization of mRNA. MicroRNA sequences have been described in publications such as Lim, et al., Genes & Development, 17, p. 991 - 1008 (2003), Lim et al Science 299, 1540 (2003), Lee and Ambros Science, 294, 862 (2001), Lau et al., Science 294, 858-861 (2001), Lagos-Quintana et al, Current Biology, 12, 735-739 (2002), Lagos Quintana et al, Science 294, 853- 857 (2001), and Lagos-Quintana et al, RNA, 9, 175- 179 (2003), which are incorporated by reference. Multiple microRNAs can also be incorporated into a precursor molecule. Furthermore, miRNA-like stem-loops can be expressed in cells as a vehicle to deliver artificial miRNAs and short interfering RNAs (siRNAs) for the purpose of modulating the expression of endogenous genes through the miRNA and or RNAi pathways.

As used herein, "double stranded RNA" or "dsRNA" refers to RNA molecules that are comprised of two strands. Double-stranded molecules include those comprised of a single RNA molecule that doubles back on itself to form a two-stranded structure. For example, the stem loop structure of the progenitor molecules from which the single- stranded miRNA is derived, called the pre-miRNA (Bartel et al. 2004. Cell 1 16:281 -297), comprises a dsRNA molecule.

As used herein, the term “promoter” means a DNA sequence that regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in cells. The term encompasses “tissue specific” promoters, i.e., promoters which effect expression of the selected DNA sequence only in specific cells (e.g., cells of a specific tissue). The term also covers so-called “leaky” promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well. The term also encompasses non-tissue specific promoters and promoters that constitutively expressed or that are inducible (i.e., expression levels can be controlled).

The terms “protein”, “polypeptide”, and “peptide” are used interchangeably herein when referring to a gene product.

“Small molecule” as used herein, is meant to refer to a composition, which has a molecular weight of less than about 5 kD and most preferably less than about 4 kD. Small molecules can be nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic (carbon-containing) or inorganic molecules. Many pharmaceutical companies have extensive libraries of chemical and/or biological mixtures, often fungal, bacterial, or algal extracts, which can be screened with any of the assays of the invention to identify compounds that modulate a bioactivity.

A “substitution”, as used herein, refers to the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively.

“Transcriptional regulatory sequence” is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. In preferred embodiments, transcription of one of the genes is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring forms of the polypeptide.

As used herein, the term “transgene” means a nucleic acid sequence (or an antisense transcript thereto) which has been introduced into a cell. A transgene could be partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can also be present in a cell in the form of an episome. A transgene

can include one or more transcriptional regulatory sequences and any other nucleic acid, such as introns, that may be necessary for optimal expression of a selected nucleic acid.

A “transgenic animal” refers to any animal, preferably a non-human mammal, bird or an amphibian, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or in vitro fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extra-chromosomally replicating DNA. In the typical transgenic animals described herein, the transgene causes cells to express a recombinant form of one of the subject polypeptide, e.g. either agonistic or antagonistic forms. However, transgenic animals in which the recombinant gene is silent are also contemplated, as for example, the FLP or CRE recombinase dependent constructs described below. Moreover, “transgenic animal” also includes those recombinant animals in which gene disruption of one or more genes is caused by human intervention, including both recombination and antisense techniques.

As used herein, the terms "treatment" and "treating" refer to an approach for obtaining beneficial or desired results including, but not limited to, therapeutic benefit and/or a prophylactic benefit. By therapeutic benefit is meant eradication or amelioration of the underlying disorder being treated. Also, a therapeutic benefit is achieved with the eradication or amelioration of one or more of the physiological symptoms associated with the underlying disorder such that an improvement is observed in the patient, notwithstanding that the patient may still be afflicted with the underlying disorder. For prophylactic benefit, the compositions may be administered to a patient at risk of developing a particular disease, or to a patient reporting one or more of the physiological symptoms of a disease, even though a diagnosis of this disease may not have been made.

A "therapeutic effect," as used herein encompasses a therapeutic benefit and/or a prophylactic benefit as described above. A prophylactic effect includes delaying or eliminating the appearance of a disease or condition, delaying or eliminating the onset of symptoms of a disease or condition, slowing, halting, or reversing the progression of a disease or condition, or any combination thereof.

The term "subject" or "patient" as used herein refers to any animal, such as a mammal, for example a human. The methods and compositions described herein can be useful in both human therapeutics and veterinary applications. In some embodiments, the patient is a mammal, and in some embodiments, the patient is human. For veterinary purposes, the terms "subject" and "patient" include, but are not limited to, farm animals

including cows, sheep, pigs, horses, and goats; companion animals such as dogs and cats; exotic and/or zoo animals; laboratory animals including mice, rats, rabbits, guinea pigs, and hamsters; and poultry such as chickens, turkeys, ducks, and geese.

III. Exemplary Embodiments

a. IBD Stem Cells Generally

One aspect of the present invention provides isolated epithelial stem cells derived from gastrointestinal biopsies from IBD patients, referred to herein as “IBD Stem Cells". Prior to the invention, intestinal stem cells from disease samples were generally considered unviable in culture and isolation of stem cells from IBD patient biopsies and stable culture and passaging of those stem cells under conditions that maintain the genotype and epigenetics of the stem cell as it existed in the biopsy had not been described.

In certain embodiments, the IBD Stem Cells are clonal, i.e., the stem cell preparation is derived from passaging a single IBD Stem Cell clone.

In certain embodiments, the progeny of an IBD Stem Cell clone remain genetically and epigenetically stable over many passages, i.e., compared to early passage (P1, P2 or P3), the stem cells are substantially genetically and epigenetically indentical, and preferably are genetically and epigenetically identical, to the early passage stem cells even after passage 10, 20, 30, 40, 50, 75 or even 100. For instance, the late passage IBD Stem Cells are substantially epigenetically identical to early passage stem cells as measured by chromatin immunoprecipitation (ChIP), which serves to monitor changes in chromatin structure, and bisulfite modification and/or CpG island microarray, which tracks changes in DNA methylation.

In certain embodiments, the late passage progeny (even after passage 10, 20, 30, 40, 50, 75 or even 100) of an IBD Stem Cell clone remain predisposed to differentiating to substantial the same epithelial cells or tissue under the same air-liquid interface conditions as the early passage (e.g., P1, P2 or P3), e.g., substantially identical as measured by principle component analysis (PCA) of gene expression, i.e., less than a 1.5 fold, even more preferably less than a 1.0 fold or even 0.5 fold in the level of expression of the 1200 most highly expressed genes by the differentiated tissues/cells with a $p<0.05$.

In certain embodiments, the late passage progeny (even after passage 10, 20, 30, 40, 50, 75 or even 100) of Crohn’s Disease stem cell clone remain predisposed to differentiating to substantial the same epithelial cells or tissue under the same air-liquid interface conditions as the early passage (e.g., P1, P2 or P3), e.g., substantially identical as measured by principle component analysis (PCA) of pCD gene expression, i.e., less than a 1.5 fold, even more preferably less than a 1.0 fold or even 0.5 fold in the level of expression of the 1200 most highly expressed genes by the differentiated tissues/cells with a $p<0.05$.

In certain embodiments, the subject IBD stem cells are derived from disease tissue samples such as biopsies from Crohn's Disease patients, including adult or pediatric patients. In other embodiments, the subject IBD stem cells are derived from disease tissue samples such as biopsies from Ulcerative Colitis patients, including adult or pediatric patients.

In certain embodiments, the subject IBD stem cells, such as the pediatric Crohn's Disease stem cells, are characterized by a gene expression profile the same or similar to the differential gene expression profiles illustrated in Figure 1B, Figure 1F, Figure 2C, Figure 2D and/or Table 3 and/or upregulated expression in a cellular pathway shown in Figure 4B, relative to epithelial stem cells derived from normal gastrointestinal tract from same area, particularly normal gastrointestinal epithelial stem cells derived from patient matched biopsies. In certain embodiments, the IBD stem cells will have a gene expression profile that is similar to expression profiles illustrated in Figure 1B, Figure 1F, Figure 2C, Figure 2D and/or Table 3 so as to provide at least a 75% confidence interval that the IBD stem cells are the same or similar to the IBD stem cells characterized in Figure 1B, Figure 1F, Figure 2C, Figure 2D and/or Table 3, and more preferably a confidence interval of at least 80%, 85%, 90% or even 95%.

In certain embodiments, the subject IBD stem cells, such as the pediatric Crohn's Disease stem cells, can be differentiated, such as in an air-liquid interface format, to produce differentiated epithelial tissue in culture that is consistent in gene expression and pathology with inflammatory bowel disease. In certain embodiments, the IBD stem cell is characterized by the ability to produce ALI-differentiated epithelial progeny having a gene expression profile the same or similar to the differential gene expression profiles illustrated in Figure 2C, Figure 2D, Figure 2E, Figure 3C, Figure 3F, Figure 4C, Figure 4E, Figure 7 and/or Figure 19 or downregulated expression of Atonal BHLH transcription factor 1 (ATOH1), relative to ALI-differentiated epithelial cells derived from epithelial stem cells derived from normal gastrointestinal tract from same area, particularly from normal gastrointestinal epithelial stem cells derived from patient matched biopsies. In certain embodiments, the IBD stem cells is characterized by the ability to produce ALI-differentiated epithelial progeny having a gene expression profile that is similar to expression profiles illustrated in Figure 2C, Figure 2D and/or Figure 19B so as to provide at least a 75% confidence interval that the differentiated epithelail cells from the subject IBD stem cells are the same or similar to the the differentiated epithelail cells from the IBD stem cells characterized in Figure 2C, Figure 2D and/or Figure 19B, and more preferably a confidence interval of at least 80%, 85%, 90% or even 95%.

In certain embodiments, the IBD stem cell is characterized by the ability to produce ALI-differentiated 3-D mucosa that substantially lack goblet cells and have little or no

expression of MUC2 (i.e., less than 10% of cells expressing detectable MUC2, and more preferably less than 5% or even 2%), and/or little or no staining by antibodies to the enteroendocrine marker CHGA or the Paneth cell marker DEFA6 (i.e., less than 10% of cells staining for DEFA6, and more preferably less than 5% or even 2%).

In addition to the defects in secretory cell differentiation, in certain embodiments the subject IBD stem cells can be characterized based on in vitro-generated epithelia having aberrant distribution pattern of glycoprotein A33 (GPA33, see Figure 3A and 3B), a protein that forms an integral part of the tight junction in the colon and whose engineered deletion in mice yields a chronic inflammatory phenotype of the gastrointestinal tract. In certain embodiments, the subject IBD stem cells can be characterized based on in vitro-generated epithelia having a high, ectopic expression of claudin 18 (CLDN18), a tight junction protein, and of V-set and immunoglobulin domain containing 1 (VSIG1), a junctional adhesion protein (see Figure 3A and 3B) relative to epithelial stem cells derived from normal gastrointestinal tract from same area, particularly normal gastrointestinal epithelial stem cells derived from patient matched biopsies.

In certain embodiments, the subject IBD stem cells can be altered epigenetic patterns relative to epithelial stem cells derived from normal gastrointestinal tract from same area, particularly normal gastrointestinal epithelial stem cells derived from patient matched biopsies. For instance, certain of the subject IBD stem cells may show extensive regions of repressive histone 3 lysine 27 trimethylation (H3K27me3) at the 5' ends of each of the HOXA and HOXB loci (such as shown in Figures 16A and 16C) and/or be characterized by the 3' portions of the HOXA, B, and C loci of those IBD stem cells showing a pattern of histone marks consistent with an overall potentiation or activation of 3' HOX genes relative to the normal gastrointestinal tract from same area, particularly normal gastrointestinal epithelial stem cells derived from patient matched biopsies.

In certain embodiments, the subject IBD stem cells are provided as pure or substantially pure cultures of IBD stem cells, the cells in the culture being at least 85% pure IBD stem cells, and even more preferably 90%, 95% or even 98% pure IBD stem cells.

In other embodiments, the invention provides pure or substantially pure cultures of epithelial cells, including in the form of 3-D mucosa, which are differentiated from the subject IBD stem cells, the cells in the differentiated culture being at least 85% pure differentiated IBD epithelial tissue, and even more preferably 90%, 95% or even 98% pure differentiated IBD epithelial tissue.

In certain embodiments, the subject Crohn's Disease (CD) stem cells are provided as pure or substantially pure cultures of IBD stem cells, the cells in the culture being at least 85% pure CD stem cells, and even more preferably 90%, 95% or even 98% pure CD stem cells.

In other embodiments, the invention provides pure or substantially pure cultures of epithelial cells, including in the form of 3-D mucosa, which are differentiated from the subject CD stem cells, the cells in the differentiated culture being at least 85% pure differentiated Crohn's Disease epithelial tissue, and even more preferably 90%, 95% or even 98% pure differentiated Crohn's Disease epithelial tissue.

The IBD stem cells and differentiated cell cultures derived therefrom can be provided in a multitude of different culture formats as will be apparent to those skilled in the art. These may include adherent and non-adherent cultures, and may be in such forms as clusters or sheets of cells and disassociated cell suspensions. In certain embodiments, such as for drug screening or other analysis, the cells or differentiated tissues are arrayed in multiwell format plates or culture dishes.

In certain embodiments, the IBD stem cells or differentiated cell cultures are cryopreserved preparations.

b. IBD Stem Cells Engineered for Animal Model and Drug Screening Assays

In certain embodiments, the IBD stem cell can be engineered in order to alter the disease phenotype, i.e., for therapeutic, drug screening or animal model uses. The cells can be engineered by addition of recombinant sequences (i.e., integrated genomically or episomally) to cause expression of recombinant gene products (proteins or RNA) or engineered to decrease or remove the expression of gene product otherwise expressed by diseases stem cell. In certain embodiments, the IBD stem cells are engineered to alter the phenotype of differentiated tissue resulting arising from the IBD stem cells to a phenotype resembling normal epithelial for the region of the gastrointestinal tract from which the IBD stem cell was isolated. In certain embodiments, the IBD stem cells are engineered to express detectable markers that are useful in drug discovery or following cell fate in animal models. For instance, the coding sequence for one or more detectable markers can be engineered in the IBD stem cell so as correlate with the proliferative capacity of the stem cell, the death (or conversely viability) of the IBD stem cell, the differentiation fate of the IBD stem cell, the proliferative capacity and/or viability of a differentiated cell derived from an IBD stem cell, the epigenetic state of the IBD stem cell or of a differentiated cell derived from the IBD stem cells.

In certain embodiments, the IBD stem cell can be engineered with coding sequences for one or more selectable traits when expressed in the IBD stem cell or differentiated cells derived therefrom. For instance, the IBD stem cell can be engineered with coding sequences for one or more drug selection traits.

In certain embodiments, the IBD stem cell can be engineered with coding sequences for one or more "suicide" genes which provide for sensitivity to culture conditions, such as

the presence or absence of a small molecule agent (drug) that causes inducible killing of the engineered cell but not the unengineered version of the cell.

For further illustration, any portion of the genome of an engineered IBD stem cells can be deleted to disrupt the expression of an endogenous IBD stem cells gene. Non-limiting examples of genomic regions that can be deleted or disrupted in the genome of IBD stem cells include a promoter, an activator, an enhancer, an exon, an intron, a non-coding RNA, a micro-RNA, a small-nuclear RNA, variable number tandem repeats (VNTRs), short tandem repeat (STRs), SNP patterns, hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, or simple sequence repeats. In some cases, the deleted a portion of the genome ranges between 1 nucleic acid to about 10 nucleic acids, 1 nucleic acid to about 100 nucleic acids, 1 nucleic acid to about 1,000 nucleic acids, 1 nucleic acid to about 10,000 nucleic acids, 1 nucleic acid to about 100,000 nucleic acids, 1 nucleic acid to about 1,000,000 nucleic acids, or other suitable range.

Various techniques known in the art can be used to introduce a cloned, or synthetically engineered, nucleic acid comprising the genetic code for a gene product of interest, sucha s protein, into a specific location within the genome of an engineered IBD stem cells. The RNA-guided Cas9 nuclease from the microbial clustered regularly interspaced short palindromic repeats (CRISPR) system, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and meganuclease technologies, as described, respectively by WO201409370, WO2003087341, WO2014134412, and WO2011090804, each of which is incorporated by reference herein in its entireties, can be used to provide efficient genome engineering in IBD stem cells(s). The technologies described herein can also be used to insert the expression cassette into a genomic location that simultaneously provides a knock-out of one gene and a knock-in of another gene.

US 5,416,260; US 5,413,923; US 5,574,205; US 6,139,835; US 6,514,752 describe an approach using homologous recombination to either inactivate a target gene, or to inactivate genes that regulate the expression of the gene of interest. Because the approach results in only the required sequence changes, the likelihood of secondary mutations is reduced. The technology provides additional methods and targeting constructs that increase the detection of low frequency homologous recombination events.

To illustrate another approach, gene editing techniques such as Cre-LoxP, TALEN and CRISPR systems can be deployed to disrupt expression of a gene of interest, or to insert the coding sequence for an exogenous gene encoding a desired gene product. In other representative embodiments, expression of a target gene can be downregulated by introducing an RNA interference construct, such as a small interfering RNA or hairpin RNA, targeting the gene of interest.

Torikai et al. Blood. 2013 Aug 22; 122(8): 1341–1349 describes a zinc finger nucleases system employing a “hit-and-run” approach to genetic editing for selective elimination of expression of a target, particular at the point of engineering the pluripotent stem cells from which they are derived. Hacke et al. Immunol Res. 2009;44(1-3):112-26 describe the suppression of gene expression by lentivirus-mediated gene transfer of siRNA cassettes which can be readily applied in the generation of the engineered IBD stem cells of the present invention. For efficient and stable delivery of short hairpin-type RNAi constructs (shRNA), those authors employed lentivirus-based gene transfer vectors that integrate into genomic DNA, thereby permanently modifying transduced donor cells. PCT Application WO2016183041 and Mesissner et al. J Immunol May 1, 2015, 194 (1 Supplement) 140.28 describe a using modern genome editing tools such as Talen and CRISPR/Cas9 system that can be used in the generation of engineered IBD stem cells pursuant to the current invention.

Such CRISPR/Cas systems can employ a variety of Cas proteins (Haft et al. PLoS Comput Biol. 2005; l(6)e60). In some embodiments, the CRISPR/Cas system is a CRISPR type I system. In some embodiments, the CRISPR/Cas system is a CRISPR type II system. In some embodiments, the CRISPR/Cas system is a CRISPR type V system. For example, CRISPR/Cas systems may be used to target transcriptional regulators of a target gene to either produce an engineered IBD cell in which expression of the target gene is downregulated or otherwise disrupted in the IBD stem cell or differentiated progeny, as well as embodiments in which the expression of the target gene is upregulated in the IBD stem cell or differentiated progeny or in which the transcriptional regulation of the target gene can be regulated by the addition of exogenous factors such as a small molecules.

In some embodiments, the alteration is an indel. As used herein, "indel" refers to a mutation resulting from an insertion, deletion, or a combination thereof. As will be appreciated by those skilled in the art, an indel in a coding region of a genomic sequence will result in a frameshift mutation, unless the length of the indel is a multiple of three. In some embodiments, the alteration is a point mutation. As used herein, "point mutation" refers to a substitution that replaces one of the nucleotides. A CRISPR/Cas system can be used to induce an indel of any length or a point mutation in a target polynucleotide sequence.

In some embodiments, the alteration results in a knock out of the target polynucleotide sequence or a portion thereof. For example, knocking out a target polynucleotide sequence in a cell can be performed in vitro, in vivo or ex vivo for both therapeutic and research purposes. Knocking out a target polynucleotide sequence in an IBD stem cell can be useful in understanding the role of that gene in an inflammatory bowel disease or if the resulting engineered cells is intended to be used therapeutically in treating or preventing a disorder associated with expression of the target polynucleotide sequence

(e.g., by knocking out a mutant allele in a cell ex vivo and introducing those cells comprising the knocked out mutant allele into a subject). As used herein, "knock out" includes deleting all or a portion of the target polynucleotide sequence in a way that interferes with the function of the target polynucleotide sequence or its expression product.

In some embodiments, the alteration results in reduced expression of the target polynucleotide sequence. The terms "decrease," "reduced," "reduction," and "decrease" are all used herein generally to mean a decrease by a statistically significant amount. However, for avoidance of doubt, "decreased," "reduced," "reduction," "decrease" includes a decrease by at least 10% as compared to a reference level, for example a decrease by at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%), or at least about 80%, or at least about 90% or up to and including a 100% decrease (i.e. absent level as compared to a reference sample), or any decrease between 10-100% as compared to a reference level.

In some embodiments, such as upregulation of tolerogenic gene products, the genome editing resulted in increased expression of a gene product. The terms "increased," "increase" or "enhance" or "activate" are all used herein to generally mean an increase by a statically significant amount; for the avoidance of any doubt, the terms "increased", "increase" or "enhance" or "activate" means an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%), or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100%) as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level.

In some embodiments, the alteration is a homozygous alteration. In some embodiments, the alteration is a heterozygous alteration.

In those embodiments intended for drug discovery, the engineered IBD stem cells (or resulting differentiated cells and tissue) can include a reporter gene which ultimately measures the end stage of a cascade of events, such as transcriptional modulation, cell death or resistance to bacterial toxins (such as Clostridium difficile toxins, i.e., TcdA and/or TcdB). A "reporter gene" includes any gene that expresses a detectable gene product, which may be RNA or protein. Preferred reporter genes are those that are readily detectable. The reporter gene may also be included in the construct in the form of a fusion gene with a gene that includes desired transcriptional regulatory sequences or exhibits other desirable properties.

Accordingly, in practicing one embodiment of the assay, a reporter gene construct is inserted into the IBD stem cell in order to generate a detection signal dependent on a desired change in the characteristics of the IBD stem cell (or progeny) or the proliferation, senescence/quiescence or death of the IBD stem cell (or progeny). Typically, the reporter gene construct will include a reporter gene in operative linkage with one or more transcriptional regulatory elements with the level of expression of the reporter gene providing a detection signal dependent on the associated transcriptional regulatory sequence(s). Many reporter genes and transcriptional regulatory elements useful in the subject engeineered cells and drug screening assays are known to those of skill in the art and others may be readily identified or synthesized.

Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), Nature 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al. (1987), Mol. Cell. Biol. 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), PNAS 1: 4154-4158; Baldwin et al. (1984), Biochemistry 23: 3663-3667); phycobiliproteins (especially phycoerythrin); green fluorescent protein (GFP: see Valdivia et al. (1996) Mol Microbiol 22: 367-78; Cornack et al. (1996) Gene 173 (1 Spec No): 33-8; and Fey et al. (1995) Gene 165:127-130; alkaline phosphatase (Toh et al. (1989) Eur. J Biochem. 182: 231-238, Hall et al. (1983) J Mol. Appl. Gen. 2: 101), secreted alkaline phosphatase (Cullen and Malim (1992) Methods in Enzymol 216:362-368). Other examples of suitable reporter genes include those which encode proteins conferring drug/antibiotic resistance to the host bacterial cell, or which encode proteins required to complement an auxotrophic phenotype. A preferred reporter gene is the spc gene, which confers resistance to spectinomycin.

The amount of transcription from the reporter gene may be measured using any method known to those of skill in the art to be suitable. For example, specific mRNA expression may be detected using Northern blots or specific protein product may be identified by a characteristic stain or an intrinsic activity.

In preferred embodiments, the gene product of the reporter is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detection signal based on color, fluorescence, or luminescence. Merely to illustrate, the amount of expression from the reporter gene is then compared to the amount of expression in either the same cell in the absence of a test compound or it may be compared with the amount of transcription in a substantially identical cell treated with a known control agent.

In other preferred embodiments, the reporter gene provides a selection method such that cells in which the reporter gene is activated have a growth advantage. For example the reporter could enhance cell viability, e.g., by relieving a cell nutritional requirement, and/or

provide resistance to a drug. For example the reporter gene could encode a gene product which confers the ability to grow in the presence of a selective agent, e.g., chorlamphenicol or kanamycin.

Another class of useful reporter genes encode cell surface proteins for which antibodies or ligands are available. Expression of the reporter gene allows cells to be detected or affinity purified by the presence of the surface protein.

In appropriate assays, so-called counterselectable or negatively selectable genes may be used.

The marker gene may also be a screenable gene. The screened characteristic may be a change in cell morphology, metabolism or other screenable features. Suitable markers include .beta.-galactosidase, alkaline phosphatase, horseradish peroxidase, luciferase, bacterial green fluorescent protein,; secreted alkaline phosphatase (SEAP); and chloramphenicol transferase (CAT).

c. Cell Cultures and Media Systems for IBD Stem Cells

The isolation, passaging and maintenance of the subject IBD stem cells can be carried out using, for example, a culture media system comprising (a) a ROCK (Rho Kinase) inhibitor; (b) a Wnt agonist; (c); a Bone Morphogenetic Protein (BMP) antagonist; (d) a Notched Inhibitor; and (e) a TGFβ signaling pathway inhibitor (e.g., a TGFβ inhibitor or a TGFβ receptor inhibitor). In certain embodiments, the the culture medium optionally further comprising: a mitogenic growth factor; and/or insulin or IGF; and/or nicotinamide or an analog thereof. In certain embodiments, the cells from the IBD tissue sample, or the repassaged IBD stem cells, are optionally in fluid or direct contact with mitotically inactive feeder cells and/or in contact with extracellular matrix (such as a basement membrane matrix) or other bio- or synthetic matrix. In the case of the isolation of IBD stem cells from tissue samples, such as biopsies, the method can be performed by culturing dissociated epithelial cells from an IBD tissue sample in the medium, isolating single cells from the epithelial cell clones that arise, and culturing the isolated single cells from to form individual cultures of single cell clones, i.e., in contact with feeder cells and/or a basement membrane matrix in the medium, where each of the single cell clones represents a clonal expansion of the IBD epithelial stem cell.

In certain embodiments, the (epithelial) cells are dissociated from the tissue sample through enzymatic digestion with an enzyme. For example, the enzyme may comprise collagenase, protease, dispase, pronase, elastase, hyaluronidase, Accutase or trypsin.

In certain embodiments, the (epithelial) cells are dissociated from the tissue sample through dissolving extracellular matrix surrounding the (epithelial) cells.

In certain embodiments, the mitotically inactivated cells are mitotically-inactivated fibroblasts, preferably human or murine fibroblasts, such as 3T3-J2 cells. Mitotic inactivation can be accomplished by the administration of mitomycin C or other chemically-based mitotic inhibitors, irradiation with γ-rays, irradiation with X-rays, and/or irradiation with UV light.

In certain embodiments, the extracellular matrix is a basement membrane matrix, such as a laminin-containing basement membrane matrix (e.g., MATRIGEL™ basement membrane matrix (BD Biosciences)), and is preferably growth factor-reduced. In other embodiments, the biopolymer is selected from the group consisting of collagen, chitosan; fibronectin, fibrin, and mixtures thereof.

In certain embodiments, the basement membrane matrix does not support 3-dimensional growth, or does not form a 3-dimensional matrix necessary to support 3-dimensional growth.

In certain embodiments, the medium further comprises serum, preferably FBS (and even more preferably FBS that is not heat inactivated), such as in a concentration of 5%-15%, such as 10% FBS.

In certain embodiments, the medium further comprises 10% FBS that is not heat inactivated.

In certain embodiments, the medium includes a mitogenic growth factor suchas EGF, Keratinocyte Growth Factor (KGF), TGFa, BDNF, HGF, and/or bFGF (e.g. , FGF7 or FGF10).

(i) Rock (Rho-kinase) Inhibitor

While not wishing to be bound by any particular theory, the addition of a Rock inhibitor may prevent anoikis, especially when culturing single stem cells. The Rock inhibitor may be (1R,4R)-4-((R)-1-aminoethyl)-N-(pyridin-4-yl)cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632, Sigma- Aldrich), 5-(1,4-diazepan-1-ylsulfonyl)isoquinoline (fasudil or HA1077, Cayman Chemical), (1S,)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]-hexahydro-1H-1,4-diazepine dihydrochloride (H- I 152, Tocris Bioscience), and N-(6-fluoro-1H-indazol-5-yl)-2-methyl-6-oxo-4-(4-(trifluoromethyl)phenyl)-1 ,4,5,6-tetrahydropyridine-3-carboxamide (GSK429286A, Stemgent).

In certain embodiments, the final concentration for Y27632 is about 1-5 μM, or 2.5 μM.

The Rho-kinase inhibitor, e.g. Y-21632, may be added to the culture medium every 1, 2, 3, 4, 5, 6, or 7 days during the first seven days of culturing the stem cells.

(ii) Wnt Agonist

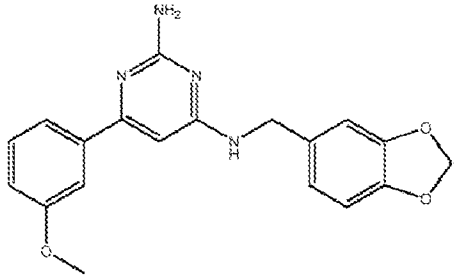
The Wnt signaling pathway is defined by a series of events that occur when a Wnt protein ligand binds to a cell- surface receptor of a Frizzled receptor family member. This results in the activation of Dishevelled (Dsh) family proteins which inhibit a complex of proteins that includes axin, GSK-3, and the protein APC to degrade intracellular β-catenin. The resulting enriched nuclear β-catenin enhances transcription by TCF/LEF family of transcription factors. A "Wnt agonist" as used herein includes an agent that directly or indirectly activates TCF/LEF-mediated transcription in a cell, such as through modulating the activity of any one of the proteins / genes in the Wnt signaling cascade (e.g., enhancing the activity of a positive regulator of the Wnt signaling pathway, or inhibiting the activity of a negative regulator of the Wnt signaling pathway).

Wnt agonists are selected from true Wnt agonists that bind and activate a Frizzled receptor family member including any and all of the Wnt family proteins, an inhibitor of intracellular β-catenin degradation, and activators of TCF/LEF. The Wnt agonist may stimulate a Wnt activity in a cell by at least about 10%, at least about 20%, at least about 30%, at least about 50%, at least about 70%, at least about 90%, at least about 100%, at least about 2-fold, 3-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold, or 1000- fold or more relative to a level of the Wnt activity in the absence of the Wnt agonist. As is known to a person of skill in the art, a Wnt activity can be determined by measuring the transcriptional activity of Wnt, for example by pTOPFLASH and pFOPFLASH Tcf luciferase reporter constructs (see Korinek et al, Science 275: 1784-1787, 1997, incorporated herein by reference).

Representative Wnt agonist may comprise a secreted glycoprotein including Wnt-1/Int-I, Wnt-2/Irp (Int-1 -related Protein), Wnt-2b/13, Wnt-3/Int-4, Wnt-3a (R&D systems), Wnt- 4, Wnt-5a, Wnt-5b, Wnt-6 (Kirikoshi et al, Biochem. Biophys. Res. Com., 283:798- 805, 2001), Wnt-7a (R&D systems), Wnt-7b, Wnt-8a/8d, Wnt-8b, Wnt-9a/14, Wnt- 9b/14b/15, Wnt- 10a, Wnt- 10b/ 12, Wnt- 11, and Wnt- 16. An overview of human Wnt proteins is provided in "The Wnt Family of Secreted Proteins," R&D Systems Catalog, 2004 (incorporated herein by reference).

Further Wnt agonists include the R-spondin family of secreted proteins, which is implicated in the activation and regulation of Wnt signaling pathway, and which comprises at least 4 members, namely R-spondin 1 (NU206, Nuvelo, San Carlos, CA), R-spondin 2 (R&D systems), R-spondin 3, and R-spondin 4. Wnt agonists also include Norrin (also known as Norrie Disease Protein or NDP) (R&D systems), which is a secreted regulatory protein that functions like a Wnt protein in that it binds with high affinity to the Frizzled-4 receptor and induces activation of the Wnt signaling pathway (Kestutis Planutis et al, BMC Cell Biol. 8: 12, 2007).

Wnt agonists further include a small-molecule agonist of the Wnt signaling pathway, an aminopyrimidine derivative (N^4-(benzo[d][1,3]dioxol-5-ylmethyl)-6-(3-methoxyphenyl)pyrimidine-2,4-diamine) of the following structure, as described in Liu et al. (Angew Chem. Int. Ed. Engl. 44 13): 1987-1990, 2005, incorporated herein by reference).



GSK-inhibitors comprise small-interfering RNAs (siRNA, Cell Signaling), lithium (Sigma), kenpaullone (Biomol International, Leost et al., Eur. J. Biochem. 267:5983-5994, 2000), 6-Bromoindirubin-30-acetoxime (Meyer et al., Chem. Biol. 10:1255-1266, 2003), SB 216763, and SB 415286 (Sigma-Aldrich), and FRAT-family members and FRAT-derived peptides that prevent interaction of GSK-3 with axin. An overview is provided by Meijer et al. (Trends in Pharmacological Sciences 25:471-480, 2004, incorporated herein by reference). Methods and assays for determining a level of GSK-3 inhibition are known in the art, and may comprise, for example, the methods and assay as described in Liao et al. (Endocrinology 145(6):2941-2949, 2004, incorporated herein by reference).

In certain embodiments, Wnt agonist is selected from: one or more of a Wnt family member, R-spondin 1-4 (such as R-spondin 1), Norrin, Wnt3a, Wnt- 6, and a GSK-inhibitor.

In certain embodiments, the Wnt agonist comprises or consists of R-spondin 1. R-spondin 1 may be added to the subject culture medium at a concentration of at least about 50 ng/mL, at least about 75 ng/mL, at least about 100 ng/mL, at least about 125 ng/mL, at least about 150 ng/mL, at least about 175 ng/mL, at least about 200 ng/mL, at least about 300 ng/mL, at least about 500 ng/mL. In certain embodiments, R-spondin 1 is about 125 ng/mL.

In certain embodiments, any of the specific protein-based Wnt agonist referenced herein, such as R-spondin 1 to R-spondin 4, any Wnt family member, etc. may be replaced by a natural, synthetic, or recombinantly produced homologs or fragments thereof that retain at least about 80%, 85%, 90%, 95%, 99% of the respective Wnt agonist activity, and/or homologs or fragments thereof that share at least about 60%, 70%, 80%, 90%, 95%, 97%, 99% amino acid sequence identity as measured by any art recognized sequence alignment software based on either a global alignment technique (e.g., the Needleman-Wunsch algorithm) or a local alignment technique (e.g., the Smith-Waterman algorithm). The

sequences of the representative Wnt agonist referenced herein are represented in SEQ ID NOs. 10 - 17.

During culturing of the subject stem cells, the Wnt family member may be added to the medium every day, every second day, every third day, while the medium is refreshed, e.g., every 1, 2, 3, 4, 5, or more days.

In certain embodiments, a Wnt agonist is selected from the group consisting of: an R-spondin, Wnt-3a and Wnt-6, or combinations thereof. In certain embodiments, an R-spondin and Wnt-3a are used together as Wnt agonist. In certain embodiments, R-spondin concentration is about 125 ng/mL, and Wnt3a concentration is about 100 ng/mL.

(iii) BMP Inhibitor

Bone Morphogenetic Proteins (BMPs) bind as a dimeric ligand to a receptor complex consisting of two different receptor serine/threonine kinases, type I and type II receptors. The type II receptor phosphorylates the type I receptor, resulting in the activation of this receptor kinase. The type I receptor subsequently phosphorylates specific receptor substrates (such as SMAD), resulting in a signal transduction pathway leading to transcriptional activity.

A BMP inhibitor as used herein includes an agent that inhibits BMP signaling through its receptors. In one embodiment, a BMP inhibitor binds to a BMP molecule to form a complex such that BMP activity is neutralized, for example, by preventing or inhibiting the binding of the BMP molecule to a BMP receptor. Examples of such BMP inhibitors may include an antibody specific for the BMP ligand, or an antigen-binding portion thereof. Other examples of such BMP inhibitors include a dominant negative mutant of a BMP receptor, such as a soluble BMP receptor that binds the BMP ligand and prevents the ligand from binding to the natural BMP receptor on the cell surface.

Alternatively, the BMP inhibitor may include an agent that acts as an antagonist or reverse agonist. This type of inhibitor binds with a BMP receptor and prevents binding of a BMP to the receptor. An example of such an agent is an antibody that specifically binds a BMP receptor and prevents binding of BMP to the antibody-bound BMP receptor.

In certain embodiments, the BMP inhibitor inhibits a BMP-dependent activity in a cell to at most 90%, at most 80%, at most 70%, at most 50%, at most 30%, at most 10%, or about 0% (near complete inhibition), relative to a level of a BMP activity in the absence of the inhibitor. As is known to one of skill in the art, a BMP activity can be determined by, for example, measuring the transcriptional activity of BMP as exemplified in Zilberberg et al. ("A rapid and sensitive bioassay to measure bone morphogenetic protein activity," BMC Cell Biology 8:41, 2007, incorporated herein by reference).

Several classes of natural BMP-binding proteins are known, including Noggin (Peprotech), Chordin, and chordin-like proteins comprising a chordin domain (R&D systems)

comprising chordin domains, Follistatin and follistatin-related proteins comprising a follistatin domain (R&D systems) comprising a follistatin domain, DAN and DAN-like proteins comprising a DAN Cystine-knot domain {e.g., Cerberus and Gremlin) (R&D systems), sclerostin / SOST (R&D systems), decorin (R&D systems), and alpha-2 macroglobulin (R&D systems) or as described in US 8,383,349. An exemplary BMP inhibitor for use in a method of the invention is selected from Noggin, DAN, and DAN-like proteins including Cerberus and Gremlin (R&D systems). These diffusible proteins are able to bind a BMP ligand with varying degrees of affinity, and inhibit BMPs' access to their signaling receptors.

Any of the above-described BMP inhibitors may be added either alone or in combination to the subject culture medium when desirable.

In certain embodiments, the BMP inhibitor is Noggin. Noggin may be added to the respective culture medium at a concentration of at least about 10 ng/mL, or at least about 20 ng/mL, or at least about 50 ng/mL, or at least about 100 ng/mL (e.g., 100 ng/mL).

In certain embodiments, any of the specific BMP inhibitors referenced herein, such as Noggin, Chordin, Follistatin, DAN, Cerberus, Gremlin, sclerostin / SOST, decorin, and alpha-2 macroglobulin may be replaced by a natural, synthetic, or recombinantly produced homologs or fragments thereof that retain at least about 80%, 85%, 90%, 95%, 99% of the respective BMP inhibiting activity, and/or homologs or fragments thereof that share at least about 60%, 70%, 80%, 90%, 95%, 97%, 99% amino acid sequence identity as measured by any art recognized sequence alignment software based on either a global alignment technique (e.g. , the Needleman-Wunsch algorithm) or a local alignment technique (e.g. , the Smith- Waterman algorithm).

The sequences of the representative BMP inhibitors referenced herein are represented in SEQ ID NOs. 1 - 9.

During culturing of the subject stem cells, the BMP inhibitor may be added to the culture medium every day, every 2nd day, every 3rd day, or every 4th day, while the culture medium is refreshed every day, every second day, every third day, or every fourth day as appropriate.

(iv) TGF-beta or TGF-beta Receptor Inhibitor

TGF-β signaling is involved in many cellular functions, including cell growth, cell fate and apoptosis. Signaling typically begins with binding of a TGF-β superfamily ligand to a Type II receptor, which recruits and phosphorylates a Type I receptor. The Type 1 receptor then phosphorylates SMADs, which act as transcription factors in the nucleus and regulate target gene expression. Alternatively, TGF-β signaling can activate MAP kinase signaling pathways, for example, via p38 MAP kinase.

The TGF-β superfamily ligands comprise bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), anti-Mullerian hormone (AMH), activin, nodal and TGF-βs.

A TGF-β inhibitor as used herein includes an agent that reduces the activity of the TGF-β signaling pathway. There are many different ways of disrupting the TGF-β signaling pathway known in the art, any of which may be used in conjunction with the subject invention. For example, TGF-β signaling may be disrupted by: inhibition of TGF-β expression by a small-interfering RNA strategy; inhibition of furin (a TGF-β activating protease); inhibition of the pathway by physiological inhibitors, such as inhibition of BMP by Noggin, DAN or DAN-like proteins; neutralization of TGF-β with a monoclonal antibody; inhibition with small-molecule inhibitors of TGF-β receptor kinase 1 (also known as activin receptor-like kinase, ALK5), ALK4, ALK6, ALK7 or other TGFβ-related receptor kinases; inhibition of Smad 2 and Smad 3 signaling by overexpression of their physiological inhibitor, Smad 7, or by using thioredoxin as an Smad anchor disabling Smad from activation (Fuchs, Inhibition of TGF-β Signaling for the Treatment of Tumor Metastasis and Fibrotic Diseases. Current Signal Transduction Therapy 6(1):29-43(15), 2011).

For example, a TGF-β inhibitor may target a serine/threonine protein kinase selected from: TGF-β receptor kinase 1, ALK4, ALK5, ALK7, or p38. ALK4, ALK5 and ALK7 are all closely related receptors of the TGF-β superfamily. ALK4 has GI number 91; ALK5 (also known as TGF-β receptor kinase 1) has GI number 7046; and ALK7 has GI number 658. An inhibitor of any one of these kinases is one that effects a reduction in the enzymatic activity of any one (or more) of these kinases. Inhibition of ALK and p38 kinase has previously been shown to be linked in B-cell lymphoma (Bakkebo et al, "TGFβ-induced growth inhibition in B-cell lymphoma correlates with Smad 1/5 signaling and constitutively active p38MAPK," BMC Immunol. 11:57, 2010).

In certain embodiments, a TGF-β inhibitor may bind to and inhibit the activity of a Smad protein, such as R-SMAD or SMAD1-5 {i.e., SMAD1, SMAD2, SMAD3, SMAD4 or SMAD5).

In certain embodiments, a TGF-β inhibitor may bind to and reduces the activity of Ser/Thr protein kinase selected from: TGF-β receptor kinase 1, ALK4, ALK5, ALK7, or p38.

In certain embodiments, the medium of the invention comprises an inhibitor of ALK5.

In certain embodiments, the TGF-β inhibitor or TGF-β receptor inhibitor does not include a BMP antagonist {i.e., is an agent other than BMP antagonist).

Various methods for determining if a substance is a TGF-β inhibitor are known. For example, a cellular assay may be used in which cells are stably transfected with a reporter construct comprising the human PAI-1 promoter or Smad binding sites, driving a luciferase reporter gene. Inhibition of luciferase activity relative to control groups can be used as a

measure of compound activity (De Gouville et al, Br. J. Pharmacol. 145(2): 166-177, 2005, incorporated herein by reference). Another example is the ALPHASCREEN® phosphosensor assay for measurement of kinase activity (Drew et al, J. Biomol. Screen. 16(2): 164-173, 2011, incorporated herein by reference).

A TGF-β inhibitor useful for the present invention may be a protein, a peptide, a small-molecule, a small-interfering RNA, an antisense oligonucleotide, an aptamer, an antibody or an antigen-binding portion thereof. The inhibitor may be naturally occurring or synthetic. Examples of small-molecule TGF-β inhibitors that can be used in the context of this invention include, but are not limited to, the small molecule inhibitors listed in Table 1A below:

Inhibitor	Targets	IC50 (nM)	Mol Wt	Name	Formula
A83-01	ALK5 (TGF-β RI)	12	421.52	3-(6-Methyl-2-pyridinyl)-N-phenyl-4-(4-quinolinyl)-1H-pyrazole-1-carbothioamide	C25H19N5S
	ALK4	45			
	ALK7	7.5			
SB-431542	ALK5	94	384.39	4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazol-2-yl]benzamide	C22H16N4O3
	ALK4				
	ALK7				
SB-505124	ALK5	47	335.4	2-(5-benzo[1,3]dioxol-5-yl-2-tert-butyl-3H-imidazol-4-yl)-6-methylpyridine hydrochloride hydrate	C20H21N3O2
	ALK4	129			
SB-525334	ALK5	14.3	343.42	6-[2-(1,1-Dimethylethyl)-5-(6-methyl-2-pyridinyl)-1H-imidazol-4-yl]quinoxaline	C21H21N5
SD-208	ALK5	49	352.75	2-(5-Chloro-2-fluorophenyl)-4-[(4-pyridylamino)pteridine	C17H10ClFN6
LY-36494	TGF-β RI	59	272.31	4-[3-(2-Pyridinyl)-1H-pyrazol-4-yl]-quinoline	C17H12N4
	TGF-β RII	400			
	MLK-7K	1400			
SJN-2511	ALK5	23	287.32	2-(3-(6-Methylpyridin-2-yl)-1H-pyrazol-4-yl)-1,5-naphthyridine	C17H13N5

Table 1A: Small-molecule TGF- inhibitors targeting receptor kinases

One or more of any of the inhibitors listed in Table 1 above, or a combination thereof, may be used as a TGF-β inhibitor in the subject invention. In certain embodiments, the combination may include: SB-525334 and SD-208 and A83-01; SD-208 and A83-01; or SD-208 and A83-01.

One of skill in the art will appreciate that a number of other small-molecule inhibitors exist that are primarily designed to target other kinases, but at high concentrations may also inhibit TGF-β receptor kinases. For example, SB-203580 is a p38 MAP kinase inhibitor that, at high concentrations (for example, approximate 10 μM or more) may inhibit ALK5. Any such inhibitor that inhibits the TGF-β signaling pathway may also be used in this invention. In certain embodiments, A83-01 may be added to the culture medium at a concentration of between 10 nM and 10 μM, or between 20 nM and 5 μM, or between 50 nM and 1 μM. In certain embodiments, A83-01 may be added to the medium at about 500 nM. In certain embodiments, A83-01 may be added to the culture medium at a concentration of between 350-650 nM, 450-550 nM, or about 500 nM. In certain embodiments, A83-01 may be added to the culture medium at a concentration of between 25-75 nM, 40-60 nM, or about 50 nM.

SB-431542 may be added to the culture medium at a concentration of between 80 nM and 80 μM, or between 100 nM and 40 μM, or between 500 nM and 10 μM, or between 1-5 μM. For example, SB-431542 may be added to the culture medium at about 2 μM.

SB-505124 may be added to the culture medium at a concentration of between 40 nM and 40 μM, or between 80 nM and 20 μM, or between 200 nM and 1 μM. For example, SB- 505124 may be added to the culture medium at about 500 nM.

SB-525334 may be added to the culture medium at a concentration of between 10 nM and 10 μM, or between 20 nM and 5 μM, or between 50 nM and 1 μM. For example, SB-525334 may be added to the culture medium at about 100 nM.

LY 364947 may be added to the culture medium at a concentration of between 40 nM and 40 μM, or between 80 nM and 20 μM, or between 200 nM and 1 μM. For example, LY 364947 may be added to the culture medium at about 500 nM.

SD-208 may be added to the culture medium at a concentration of between 40 nM and 40 μM, or between 80 nM and 20 μM, or between 200 nM and 1 μM. For example, SD-208 may be added to the culture medium at abut 500 nM.

S JN 2511 may be added to the culture medium at a concentration of between 20 nM and 20 μM, or between 40 nM and 10 μM, or between 100 nM and 1 μM. For example, A83-01 may be added to the culture medium at approximately 200 nM.

(v) Notch Agonist

Notch signaling has been shown to play an important role in cell-fate determination, as well as in cell survival and proliferation. Notch receptor proteins can interact with a number of surface-bound or secreted ligands, including but not limited to Jagged-1, Jagged-2, Delta-1 or Delta-like 1, Delta-like 3, Delta-like 4, etc. Upon ligand binding, Notch receptors are activated by serial cleavage events involving members of the ADAM protease family, as well as an intramembranous cleavage regulated by the gamma secretase presenilin. The

result is a translocation of the intracellular domain of Notch to the nucleus, where it transcriptionally activates downstream genes.

A "Notch agonist" as used herein includes a molecule that stimulates a Notch activity in a cell by at least about 10%, at least about 20%, at least about 30%, at least about 50%, at least about 70%, at least about 90%, at least about 100%, at least about 3-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold, 1000-fold or more, relative to a level of a Notch activity in the absence of the Notch agonist. As is known in the art, Notch activity can be determined by, for example, measuring the transcriptional activity of Notch, by a 4xwtCBF1-luciferase reporter construct described by Hsieh et al. (Mol. Cell. Biol. 16:952-959, 1996, incorporated herein by reference).

In certain embodiments, the Notch agonist is selected from: Jagged-1, Delta-1 and Delta-like 4, or an active fragment or derivative thereof. In certain embodiments, the Notch agonist is DSL peptide (Dontu et al., Breast Cancer Res., 6:R605-R615, 2004), having the amino acid sequence CDDYYYGFGCNKFCRPR (SEQ ID NOs: 36). The DSL peptide (ANA spec) may be used at a concentration between 10 μM and 100 nM, or at least 10 μM and not higher than 100 nM. In certain embodiments, the final concentration of Jagged-1 is about 0.1-10 μM; or about 0.2-5 μM; or about 0.5-2 μM; or about 1 μM.

In certain embodiments, any of the specific Notch agonist referenced herein, such as Jagged-1, Jagged-2, Delta-1 and Delta-like 4 may be replaced by a natural, synthetic, or recombinantly produced homologs or fragments thereof that retain at least about 80%, 85%, 90%, 95%, 99% of the respective Notch agonist activity, and/or homologs or fragments thereof that share at least about 60%, 70%, 80%, 90%, 95%, 97%, 99% amino acid sequence identity as measured by any art recognized sequence alignment software based on either a global alignment technique (e.g., the Needleman-Wunsch algorithm) or a local alignment technique (e.g., the Smith-Waterman algorithm).

The sequences of the representative Notch agonists referenced herein are represented in SEQ ID NOs. 28-35.

The Notch agonist may be added to the culture medium every 1, 2, 3, or 4 days during the first 1-2 weeks of culturing the stem cells.

(vi) Nicotinamide

The culture medium of the invention may additionally be supplemented with nicotinamide or its analogs, precursors, or mimics, such as methyl-nicotinamid, benazamid, pyrazinamide, thymine, or niacin. Nicotinamide may be added to the culture medium to a final concentration of between 1 and 100 mM, between 5 and 50 mM, or preferably between 5 and 20 mM. For example, nicotinamide may be added to the culture medium to a final

concentration of approximately 10 mM. The similar concentrations of nicotinamide analogs, precursors, or mimics can also be used alone or in combination.

(vii) p38 inhibitor

A "p38 inhibitor" may be included in the media, in addition to or as part of a TGF-β inhibitory function, and include an inhibitor that, directly or indirectly, negatively regulates p38 signaling, such as an agent that binds to and reduces the activity of at least one p38 isoform. p38 protein kinases (see, GI number 1432) are part of the family of mitogen-activated protein kinases (MAPKs). MAPKs are serine/threonine- specific protein kinases that respond to extracellular stimuli, such as environmental stress and inflammatory cytokines, and regulate various cellular activities, such as gene expression, differentiation, mitosis, proliferation, and cell survival/apoptosis. The p38 MAPKs exist as α, β, β2, γ and δ isoforms.

Various methods for determining if a substance is a p38 inhibitor are known, such as: phospho-specific antibody detection of phosphorylation at Thrl80/Tyrl82, which provides a well-established measure of cellular p38 activation or inhibition; biochemical recombinant kinase assays; tumor necrosis factor alpha (TNFa) secretion assays; and DiscoverRx high throughput screening platform for p38 inhibitors. Several p38 activity assay kits also exist (e.g. Millipore, Sigma- Aldrich).

In certain embodiments, high concentrations (e.g., more than 100 nM, or more than 1 μM, more than 10 μM, or more than 100 μM) of a p38 inhibitor may have the effect of inhibiting TGF-β. In other embodiments, the p38 inhibitor does not inhibit TGF-β signaling.

Various p38 inhibitors are known in the art (for example, see Table 2). In some embodiments, the inhibitor that directly or indirectly negatively regulates p38 signaling is selected from the group consisting of SB-202190, SB-203580, VX-702, VX-745, PD-169316, RO-4402257 and BIRB-796.

In certain embodiments, the medium comprises both: a) an inhibitor that binds to and reduces the activity of any one or more of the kinases from the group consisting of: ALK4, ALK5 and ALK7; and b) an inhibitor that binds to and reduces the activity of p38.

In certain embodiments, the medium comprises an inhibitor that binds to and reduces the activity of ALK5 and an inhibitor that binds to and reduces the activity of p38.

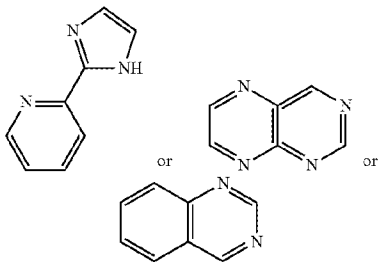
In one embodiment, the inhibitor binds to and reduces the activity of its target (for example, TGF-β and/or p38) by more than 10%; more than 30%; more than 60%; more than 80%; more than 90%; more than 95%; or more than 99% compared to a control, as assessed by a cellular assay. Examples of cellular assays for measuring target inhibition are well known in the art as described above.

An inhibitor of TGF-β and/or p38 may have an IC50 value equal to or less than 2000 nM; less than 1000 nM; less than 100 nM; less than 50 nM; less than 30 nM; less than 20 nM or less than 10 nM. The IC50 value refers to the effectiveness of an inhibitor in inhibiting its target's biological or biochemical function. The IC50 indicates how much of a particular inhibitor is required to inhibit a kinase by 50%. IC50 values can be calculated in accordance with the assay methods set out above. An inhibitor of TGF-β and/or p38 may exist in various forms, including natural or modified substrates, enzymes, receptors, small organic molecules, such as small natural or synthetic organic molecules of up to 2000 Da, preferably 800 Da or less, peptidomimetics, inorganic molecules, peptides, polypeptides, antisense oligonucleotides aptamers, and structural or functional mimetics of these including small molecules.

In certain embodiments, the inhibitor of TGF-β and/or p38 may also be an aptamer. As used herein, the term "aptamer" refers to strands of oligonucleotides (DNA or RNA) that can adopt highly specific three-dimensional conformations. Aptamers are designed to have high binding affinities and specificities towards certain target molecules, including extracellular and intracellular proteins. Aptamers may be produced using, for example, Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process (see, for example, Tuerk and Gold, Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA Polymerase. Science 249:505-510, 1990, incorporated herein by reference).

In certain embodiments, the TGF-β and/or p38 inhibitor may be a small synthetic molecule with a molecular weight of between 50 and 800 Da, between 80 and 700 Da, between 100 and 600 Da, or between 150 and 500 Da.

In certain embodiments, the TGF-β and/or p38 inhibitor comprises a pyridinylimidazole or a 2,4-disubstituted teridine or a quinazoline, for example comprises:



Particular examples of TGF-β and/or p38 inhibitors that may be used in accordance with the invention include, but are not limited to: SB-202190, SB-203580, SB-206718, SB-227931, VX-702, VX-745, PD-169316, RO-4402257, BIRB-796, A83-01 SB-431542, SB-505124, SB-525334, LY 364947, SD-208, SJ 2511 (see Table 2).

For example, SB-202190 may be added to the culture medium at a concentration of between 50 nM and 100 μM, or between 100 nM and 50 μM, or between 1 μM and 50 μM. For example, SB-202190 may be added to the culture medium at approximately 10 μM.

SB-203580 may be added to the culture medium at a concentration of between 50 nM and 100 μM, or between 100 nM and 50 μM, or between 1 μM and 50 μM. For example, SB-203580 may be added to the culture medium at approximately 10 μM.

VX-702 may be added to the culture medium at a concentration of between 50 nM and 100 μM, or between 100 nM and 50 μM, or between 1 μM and 25 μM. For example, VX-702 may be added to the culture medium at approximately 5 μM.

VX-745 may be added to the culture medium at a concentration of between 10 nM and 50 μM, or between 50 nM and 50 μM, or between 250 nM and 10 μM. For example, VX-745 may be added to the culture medium at approximately 1 μM.

PD-169316 may be added to the culture medium at a concentration of between 100 nM and 200 μM, or between 200 nM and 100 μM, or between 1 μM and 50 μM. For example, PD- 169316 may be added to the culture medium at approximately 20 μM.

RO-4402257 may be added to the culture medium at a concentration of between 10 nM and 50 μM, or between 50 nM and 50 μM, or between 500 nM and 10 μM. For example, RO-4402257 may be added to the culture medium at approximately 1 μM.

BIRB-796 may be added to the culture medium at a concentration of between 10 nM and 50 μM, or between 50 nM and 50 μM, or between 500 nM and 10 μM. For example, BIRB-796 may be added to the culture medium at approximately 1 μM.

Table 2: Exemplary TGF-β and/or p38 Inhibitors

Exemplary TGF-β and/or p38 Inhibitors

Inhibitor	Targets	IC50 (nM)	Mol Wt	Name	Formula
A83-01	ALK5 (TGF-βRI) ALK4 ALK7	12 45 7.5	421.52	3-(6-Methyl-2-pyridinyl)-N-phenyl-4-(4-quinolinyl)-1H-pyrazole-1-carbothioamide	C25H19N5S
SB-431542	ALK5 ALK4 ALK7	94	384.39	4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazol-2-yl]benzamide	C22H16N4O3
SB-505124	ALK5 ALK4	47 129	335.4	2-(5-benzo[1,3]dioxol-5-yl-2-tert-butyl-3Himidazol-4-yl)-6-methylpyridine hydrochloride hydrate	C20H21N3O2
SB-525334	ALK5	14.3	343.42	6-[2-(1,1-Dimethylethyl)-5-(6-methyl-2-pyridinyl)-1H-imidazol-4-yl]quinoxaline	C21H21N5
SD-208	ALK5	49	352.75	2-(5-Chloro-2-fluorophenyl)-4-[(4-pyridyl)amino]pteridine	C17H10ClFN6
LY-36494	TGR-βRI TGF-βRII MLK-7K	59 400 1400	272.31	4-[3-(2-Pyridinyl)-1H-pyrazol-4-yl]-quinoline	C17H12N4
LY364947	ALK5	59	272.30	4-[3-(2-pyridinyl)-1H-pyrazol-4-yl]-quinoline	$C_{17}H_{12}N_4$
SJN-2511	ALK5	23	287.32	2-(3-(6-Methylpyridine-2-yl)-1H-pyrazol-4-yl)-1,5-naphthyridine	C17H13N5
SB-202190	p38 MAP kinase p38α p38β	38 50 100	331.35	4-[4-(4-Fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]phenol	C20H14N3OF
SB-203580	p38 p38β2	50 500	377.44	4-[5-(4-Fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1H-imidazol-4-yl]pyridine	C21H16FN3OS
VX-702	p38α p38β	4-20; (Kd = 3.7) Kd = 17	404.32	6-[(Aminocarbonyl)(2,6-difluorophenyl)amino]-2-(2,4-difluorophenyl)-3-pyridinecarboxamide	C19H12F4N4O2
VX-745	p38α	10	436.26	5-(2,6-Dichlorophenyl)-2-[2,4-difluorophenyl)thio]-6H-pyrimido[1,6-b]pyridazin-6-one	C19H9Cl2F2N3OS

PD-169316	p38	89	360.3	4-[5-(4-fluorophenyl)-2-(4-nitrophenyl)-1H-imidazol-4-yl]-pyridine	C20H13FN4O
RO-4402257	p38α p38β	14 480		Pyrido[2,3-d]pyrimidin-7(8H)-one,6-(2,4-difluorophenoxy)-2-[[3-hydroxy-1-(2-hydroxyethyl)propyl]amino]-8-methyl-	
BIRB-796	p38	4	527.67	1-[2-(4-methylphenyl)-5-tert-butyl-pyrazol-3-yl]-3-[4-(2-morpholin-4-ylethoxy)naphthalen-1-yl]urea ::3-[2-(4-methylphenyl)-5-tert-butyl-pyrazol-3-yl]-1-[4-(2-morpholin-4-ylethoxy)naphthalen-1-yl]urea ::3-[3-tert-butyl-1-(4-methylphenyl)-1H-pyrazol-5-yl]-1-{4-[2-(morpholin-4-yl)ethoxy]naphthalen-1-yl}urea	C31H37N5O3

Thus, in some embodiments, the inhibitor that directly or indirectly, negatively regulates TGF-β and/or p38 signaling is added to the culture medium at a concentration of between 1 nM and 100 μM, between 10 nM and 100 μM, between 100 nM and 10 μM, or about 1 μM. For example, wherein the total concentration of the one or more inhibitor is between 10 nM and 100 μM, between 100 nM and 10 μM, or about 1 μM.

(viii) Mitogenic Growth Factors

Mitogenic growth factors suitable for the invention may include a family of growth factors comprising epidermal growth factor (EGF) (Peprotech), Transforming Growth Factor-α (TGFa, Peprotech), basic Fibroblast Growth Factor (bFGF, Peprotech), brain-derived neurotrophic factor (BDNF, R&D Systems), and Keratinocyte Growth Factor (KGF, Peprotech).

EGF is a potent mitogenic factor for a variety of cultured ectodermal and mesodermal cells, and has a profound effect on the differentiation of specific cells in vivo and in vitro, and of some fibroblasts in cell culture. The EGF precursor exists as a membrane-bound molecule, which is proteolytically cleaved to generate the 53-amino acid peptide hormone that stimulates cells. EGF may be added to the subject culture medium at a concentration of between 1-500 ng/mL. In certain embodiments, final EGF concentration in the medium is at least about 1, 2, 5, 10, 20, 25, 30, 40, 45, or 50 ng/mL, and is not higher than about 500, 450, 400, 350, 300, 250, 200, 150, 100, 50, 30, 20 ng/mL. In certain embodiments, final EGF

concentration is about 1-50 ng/mL, or about 2-50 ng/mL, or about 5-30 ng/mL, or about 5-20 ng/mL, or about 10 ng/mL.

The same concentrations may be used for an FGF, such as FGF10 or FGF7. If more than one FGF is used, for example FGF7 and FGF 10, the concentration of FGF above may refer to the total concentration of all FGF used in the medium.

In certain embodiments, any of the specific mitogenic growth factors referenced herein, such as EGF, TGFa, bFGF, BDNF, KGF, etc. may be replaced by a natural, synthetic, or recombinantly produced homologs or fragments thereof that retain at least about 80%, 85%, 90%, 95%, 99% of the respective mitogenic growth factor activity, and/or homologs or fragments thereof that share at least about 60%, 70%, 80%, 90%, 95%, 97%, 99% amino acid sequence identity as measured by any art recognized sequence alignment software based on either a global alignment technique (e.g. , the Needleman-Wunsch algorithm) or a local alignment technique (e.g. , the Smith-Waterman algorithm).

The sequences of the representative mitogenic growth factors referenced herein are represented in SEQ ID NOs. 18 - 27.

During culturing of the subject stem cells, the mitogenic growth factor may be added to the culture medium every day, every 2nd day, while the culture medium is refreshed, e.g. , every day.

Any member of the bFGF family may be used. In certain embodiments, FGF7 and/or FGF10 is used. FGF7 is also known as KGF (Keratinocyte Growth Factor). In certain embodiments, a combination of mitogenic growth factors, such as EGF and KGF, or EGF and BDNF, is added to the subject culture medium. In certain embodiments, a combination of mitogenic growth factors, such as EGF and KGF, or EGF and FGF10, is added to the subject culture medium.

(ix) Extracellular Matrix (ECM)

Extracellular matrix (ECM), used interchangeably herein with "basement membrane matrix," is secreted by connective tissue cells, and comprises a variety of polysaccharides, water, elastin, and proteins that may comprise proteoglycans, collagen, entactin (nidogen), fibronectin, fibrinogen, fibrillin, laminin, and hyaluronic acid. ECM may provide the suitable substrate and microenvironment conductive for selecting and culturing the subject stem cells.

In certain embodiments, the subject stem cells are attached to or in contact with an ECM. Different types of ECM are known in the art, and may comprise different compositions including different types of proteoglycans and/or different combination of proteoglycans. The ECM may be provided by culturing ECM -producing cells, such as certain fibroblast cells. Examples of extracellular matrix -producing cells include

chondrocytes that mainly produce collagen and proteoglycans; fibroblast cells that mainly produce type IV collagen, laminin, interstitial procollagens, and fibronectin; and colonic myofibroblasts that mainly produce collagens (type I, III, and V), chondroitin sulfate proteoglycan, hyaluronic acid, fibronectin, and tenascin-C.

In certain embodiments, at least some ECM is produced by the murine 3T3-J2 clone, which may be grown on top of the MATRIGEL™ basement membrane matrix (BD Biosciences) as feeder cell layer.

Alternatively, the ECM may be commercially provided. Examples of commercially available extracellular matrices are extracellular matrix proteins (Invitrogen) and MATRIGEL™ basement membrane matrix (BD Biosciences). The use of an ECM for culturing stem cells may enhance long-term survival of the stem cells and/or the continued presence of undifferentiated stem cells. An alternative may be a fibrin substrate or fibrin gel - or a scaffold, such as glycerolized allografts that are depleted from the original cells.

In certain embodiments, the ECM for use in a method of the invention comprises at least two distinct glycoproteins, such as two different types of collagen or a collagen and laminin. The ECM may be a synthetic hydrogel extracellular matrix, or a naturally occurring ECM. In certain embodiments, the ECM is provided by MATRIGEL™ basement membrane matrix (BD Biosciences), which comprises laminin, entactin, and collagen IV.

(x) Basal Medium

A cell culture medium that is used in a method of the invention may comprise any basal cell culture medium, such as culture medium buffered at about pH 7.4 (e.g. , between about pH 7.2-7.6) with a carbonate-based buffer. Many commercially available tissue culture media are potentially suitable for the methods of the invention, including, but are not limited to, Dulbecco' s Modified Eagle Media (DMEM, e.g. , DMEM without L-glutamine but with high glucose), Minimal Essential Medium (MEM), Knockout-DMEM (KO-DMEM), Glasgow Minimal Essential Medium (G-MEM), Basal Medium Eagle (BME), DMEM/Ham' s F12, Advanced DMEM/Ham' s F12, Iscove' s Modified Dulbecco's Media and Minimal Essential Media (MEM), Ham's F- 10, Ham' s F-12, Medium 199, cFAD and RPMI 1640 Media.

In certain embodiments, the epithelial stem cell culture system is cFAD , which consists of DMEM and Ham’s F12 (Life Technologies, CH) medium (e.g., v/v 3:1), supplemented with, e.g., 20% of fetal bovine serum (FBS) (Life Technologies, Australia), adenine (e.g., 24.3 µg/mL, Merck, CH), insulin (e.g., 5 µg/mL, Sigma, CH), 3,3,5-triiodo-L-thyronine (T3) (e.g., 2nM, Sigma, CH), hydrocortisone (e.g., 0.4 µg/mL, Sigma, CH), cholera toxin (e.g., 0.1nM, Sigma, CH), and, e.g., 1% penicillin/streptomycin (Life Technologies, CH).

The cells may be cultured in an atmosphere comprising between 5-10% C02 (e.g. , at least about 5% but no more than 10% C02, or about 5% C02). In certain embodiments,

the cell culture medium is DMEM/F12 (e.g. , 3: 1 mixture) or RPMI 1640, supplemented with L-glutamine, insulin, Penicillin/streptomycin, and/or transferrin. In certain embodiments, Advanced DMEM/F12 or Advanced RPMI is used, which is optimized for serum free culture and already includes insulin. The Advanced DMEM/F12 or Advanced RPMI medium may be further supplemented with L-glutamine and Penicillin/streptomycin. In certain embodiments, the cell culture medium is supplemented with one or more a purified, natural, semi- synthetic and/or synthetic factors described herein. In certain embodiments, the cell culture medium is supplemented by about 10% fetal bovine serum (FBS) that is not heat inactivated prior to use. Additional supplements, such as, for example, B-27® Serum Free Supplement (Invitrogen), N-Acetylcysteine (Sigma) and/or N2 serum free supplement (Invitrogen), or Neurobasal (Gibco), TeSR (StemGent) may also be added to the medium.

In certain embodiments, the medium may contain one or more antibiotics to prevent contamination (such as Penicillin/streptomycin). In certain embodiments, the medium may have an endotoxin content of less that 0.1 endotoxin units per mL, or may have an endotoxin content less than 0.05 endotoxin units per mL. Methods for determining the endotoxin content of culture media are known in the art.

A cell culture medium according to the invention allows the survival and/or proliferation and/or differentiation of epithelial stem cells on an extracellular matrix. The term "cell culture medium" as used herein is synonymous with "medium," "culture medium," or "cell medium."

The modified (growth) medium of the invention comprises, in a base medium, (a) a ROCK (Rho Kinase) inhibitor; (b) a Wnt agonist; (c) a mitogenic growth factor; (d) a TGF-beta signaling pathway inhibitor, such as TGF-beta inhibitor, or a TGF-beta receptor inhibitor); and (e) insulin or IGF; and the medium optionally further comprising a Bone Morphogenetic Protein (BMP) antagonist.

Thus in one aspect, the invention provides a base medium (Base Medium) comprising: insulin or an insulin-like growth factor; T3 (3,3 ',5-Triiodo-L- Thyronine); hydrocortisone; adenine; EGF; and 10% fetal bovine serum (without heat inactivation), in DMEM:F12 3: 1 medium supplemented with L-glutamine.

In certain embodiments, the Base Medium comprises about: 5 µg/mL insulin; 2 x 10"9 M T3 (3,3',5-Triiodo-L- Thyronine); 400 ng/mL hydrocortisone; 24.3 µg/mL adenine; 10 ng/mL EGF; and 10% fetal bovine serum (without heat inactivation), in DMEM:F12 3: 1 medium supplemented with 1.35 mM L-glutamine.

In certain embodiments, the concentration for each of the medium components referenced in the immediate preceding paragraph is independently 2%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90%, 95% higher or lower than the respective recited value, or 2-fold, 3-fold, 5-fold, 10-fold, 20-fold higher than the respective

recited value. For example, in an illustrative medium, insulin concentration may be 6 µg/mL (20% higher than the recited 5 µg/mL), EGF concentration may be 5 ng/mL (50% lower than the recited 10 ng/mL), while the remaining components each has the same concentration recited above.

In a related aspect, the invention provides a base medium containing cholera enterotoxin. In other embodiments, the base medium does not contain cholera enterotoxin.

The Base Medium may further comprise one or more antibiotics, such as Pen/Strep, and/or gentamicin.

The base media may be used to produce Modified Growth Medium (or simply Modified Medium) by adding one or more of the factors above.

(xi) Protein Sequences of the Representative Medium Factors

Several representative (non-limiting) protein factors used in the media and methods of the invention are provided below. For each listed factor, numerous homologs or functional equivalents are known in the art, and can be readily retrieved from public databases such as GenBank, EMBL, and/or NCBI RefSeq, just to name a few. Additional proteins or peptide fragments thereof, or polynucleotides encoding the same, including functional homologs from human or non-human mammals, can be readily retrieved from public sources through, for example, sequence-based searches such as NCBI BLASTp or BLASTn or both.

BMP inhibitors

Noggin: (GenBank: AAA83259.1), Homo sapiens:

(SEQ ID NO: 1)

MERCPSLGVT LYALVVVLGL RATPAGGQHY LHIRPAPSDN LPLVDLIEHP DPIFDPKEKD
LNETLLRSLL GGHYDPGFMA TSPPEDRPGG GGGAAGGAED LAELDQLLRQ
RPSGAMPSEI KGLEFSEGLA QGKKQRLSKK LRRKLQMWLW SQTFCPVLYA
WNDLGSRFWP RYVKVGSCFS KRSCSVPEGM VCKPSKSVHL TVLRWRCQRR
GGQRCGWIPI QYPIISECKC SC

Chordin (GenBank: AAG35767.1), Homo sapiens:

(SEQ ID NO 2)

MPSLPAPPAP LLLLGLLLLG SRPARGAGPE PPVLPIRSEK EPLPVRGAAG CTFGGKVYAL
DETWHPDLGE PFGVMRCVLC ACEAPQWGRR TRGPGRVSCK NIKPECPTPA
CGQPRQLPGH CCQTCPQERS SSERQPSGLS FEYPRDPEHR SYSDRGEPGA
EERARGDGHT DFVALLTGPR SQAVARARVS LLRSSLRFSI SYRRLDRPTR
IRFSDSNGSV LFEHPAAPTQ DGLVCGVWRA VPRLSLRLLR AEQLHVALVT
LTHPSGEVWG PLIRHRALAA ETFSAILTLE GPPQQGVGGI TLLTLSDTED SLHFLLLFRG

LLEPRSGGLT QVPLRLQILH QGQLLRELQA NVSAQEPGFA EVLPNLTVQE
MDWLVLGELQ MALEWAGRPG LRI SGHIAAR KSCDVLQSVL CGADALIPVQ
TGAAGSASLT LLGNGSLIYQ VQVVGTSSEV VAMTLETKPQ RRDQRTVLCH
MAGLQPGGHT AVGICPGLGA RGAHMLLQNE LFLNVGTKDF PDGELRGHVA
ALPYCGHSAR HDTLPVPLAG ALVLPPVKSQ AAGHAWLSLD THCHLHYEVL
LAGLGGSEQG TVTAHLLGPP GTPGPRRLLK GFYGSEAQGV VKDLEPELLR
HLAKGMASLL ITTKGSPRGE LRGQVHIANQ CEVGGLRLEA AGAEGVRALG
APDTASAAPP VVPGLPALAP AKPGGPGRPR DPNTCFFEGQ QRPHGARWAP
NYDPLCSLCT CQRRTVICDP VVCPPPSCPH PVQAPDQCCP VCPEKQDVRD
LPGLPRSRDP GEGCYFDGDR SWRAAGTRWH PVVPPFGLIK CAVCTCKGGT
GEVHCEKVQC PRLACAQPVR VNPTDCCKQC PVGSGAHPQL GDPMQADGPR
GCRFAGQWFP ESQSWHPSVP PFGEMSCITC RCGAGVPHCE RDDCSLPLSC
GSGKESRCCS RCTAHRRPAP ETRTDPELEK EAEGS

Follistatin (GenBank: AAH04107.1) Homo sapiens:
(SEQ ID NO: 3)
MVRARHQPGG LCLLLLLLCQ FMEDRSAQAG NCWLRQAKNG RCQVLYKTEL
SKEECCSTGR LSTSWTEEDV NDNTLFKWMI FNGGAPNCIP CK-ETCENVDC
GPGKKCRMNK KNKPRCVCAP
DCSNITWKGP VCGLDGKTYR NECALLKARC KEQPELEVQY QGRCKKTCRD
VFCPGSSTCV VDQTNNAYCV TCNRICPEPA SSEQYLCGND GVTYSSACHL
RKATCLLGRS IGLAYEGKCI KAKSCEDIQC TGGKKCLWDF KVGRGRCSLC
DELCPDSKSD EPVCASDNAT YASECAMKEA ACSSGVLLEV KHSGSCNSIS
EDTEEEEEDE DQDYSFPISS ILEW

DAN (GenBank: BAA92265.1) Homo sapiens:
(SEQ ID NO: 4)
MLRVLVGAVL PAMLLAAPPP INKLALFPDK SAWCEAKNIT QIVGHSGCEA KSIQNRACLG
QCFSYSVPNT FPQSTESLVH CDSCMPAQSM WEIVTLECPG HEEVPRVDKL
VEKILHCSCQ ACGKEPSHEG LSVYVQGEDG PGSQPGTHPH PHPHPHPGGQ
TPEPEDPPGA PHTEEEGAED

Cerberus (NCBI Reference Sequence: NP_005445.1) Homo sapiens:
(SEQ ID NO: 5)
MHLLLFQLLV LLPLGKTTRH QDGRQNQSSL SPVLLPRNQR ELPTGNHEEA
EEKPDLFVAV PHLVATSPAG EGQRQREKML SRFGRFWKKP EREMHPSRDS
DSEPFPPGTQ SLIQPIDGMK MEKSPLREEA KKFWHHFMFR KTPASQGVIL

PIKSHEVHWE TCRTVPFSQT ITHEGCEKVV VQNNLCFGKC GSVHFPGAAQ
HSHTSCSHCL PAKFTTMHLP LNCTELSSVI KVVMLVEECQ CKVKTEHEDG
HILHAGSQDS FIPGVSA

Gremlin (GenBank: AAF06677.1) Homo sapiens:
(SEQ ID NO: 6)
MSRTAYTVGA LLLLLGTLLP AAEGKKKGSQ GAIPPPDKAQ HNDSEQTQSP
QQPGSRNRGR GQGRGTAMPG EEVLESSQEA LHVTERKYLK RDWCKTQPLK
QTIHEEGCNS RTI INRFCYG QCNSFYIPRH IRKEEGSFQS CSFCKPKKFT TMMVTLNCPE
LQPPTKKKRV TRVKQCRCIS IDLD

Sclerostin/SOST (GenBank: AAK13451.1) Homo sapiens:
(SEQ ID NO: 7)
MQLPLALCLV CLLVHTAFRV VEGQGWQAFK NDATEI IPEL GEYPEPPPEL
ENNKTMNRAE NGGRPPHHPF ETKDVSEYSC RELHFTRYVT DGPCRSAKPV
TELVCSGQCG PARLLPNAIG RGKWWRPSGP DFRCIPDRYR AQRVQLLCPG
GEAPRARKVR LVASCKCKRL TRFHNQSELK DFGTEAARPQ KGRKPRPRAR
SAKANQAELE NAY

Decorin (GenBank: AAB60901.1) Homo sapiens:
(SEQ ID NO: 8)
MKATI ILLLL AQVSWAGPFQ QRGLFDFMLE DEASGIGPEV PDDRDFEPSL
GPVCPFRCQC HLRVVQCSDL

alpha-2 macroglobulin (GenBank: EAW88590.1) Homo sapiens:
(SEQ ID NO 9)
MGKNKLLHPS LVLLLLVLLP TDASVSGKPQ YMVLVPSLLH TETTEKGCVL LSYLNETVTV
SASLESVRGN RSLFTDLEAE NDVLHCVAFA VPKSSSNEEV MFLTVQVKGP
TQEFKKRTTV MVKNEDSLVF VQTDKSIYKP GQTVKFRVVS MDENFHPLNE LIPLVYIQDP
KGNRIAQWQS FQLEGGLKQF SFPLSSEPFQ GSYKVVVQKK SGGRTEHPFT
VEEFVLPKFE VQVTVPKIIT ILEEEMNVSV CGLYTYGKPV PGHVTVSICR KYSDASDCHG
EDSQAFCEKF SGQLNSHGCF YQQVKTKVFQ LKRKEYEMKL HTEAQIQEEG
TVVELTGRQS SEITRTITKL SFVKVDSHFR QGIPFFGQVR LVDGKGVPIP NKVIFIRGNE
ANYYSNATTD EHGLVQFSIN TTNVMGTSLT VRVNYKDRSP CYGYQWVSEE
HEEAHHTAYL VFSPSKSFVH LEPMSHELPC GHTQTVQAHY ILNGGTLLGL KKLSFYYLIM
AKGGIVRTGT HGLLVKQEDM KGHFSISIPV KSDIAPVARL LIYAVLPTGD VIGDSAKYDV
ENCLANKVDL SFSPSQSLPA SHAHLRVTAA PQSVCALRAV DQSVLLMKPD

AELSASSVYN LLPEKDLTGF PGPLNDQDDE DCINRHNVYI NGITYTPVSS TNEKDMYSFL
EDMGLKAFTN SKIRKPKMCP QLQQYEMHGP EGLRVGFYES DVMGRGHARL
VHVEEPHTET VRKYFPETWI WDLVVVNSAG VAEVGVTVPD TITEWKAGAF
CLSEDAGLGI SSTASLRAFQ PFFVELTMPY SVIRGEAFTL KATVLNYLPK CIRVSVQLEA
SPAFLAVPVE KEQAPHCICA NGRQTVSWAV TPKSLGNVNF TVSAEALESQ
ELCGTEVPSV PEHGRKDTVI KPLLVEPEGL EKETTFNSLL CPSGGEVSEE LSLKLPPNVV
EESARASVSV LGDILGSAMQ NTQNLLQMPY GCGEQNMVLF APNIYVLDYL
NETQQLTPEI KSKAIGYLNT GYQRQLNYKH YDGSYSTFGE RYGRNQGNTW
LTAFVLKTFA QARAYIFIDE AHITQALIWL SQRQKDNGCF RSSGSLLNNA IKGGVEDEVT
LSAYITIALL EIPLTVTHPV VRNALFCLES AWKTAQEGDH GSHVYTKALL AYAFALAGNQ
DKRKEVLKSL NEEAVKKDNS VHWERPQKPK APVGHFYEPQ APSAEVEMTS
YVLLAYLTAQ PAPTSEDLTS ATNIVKWITK QQNAQGGFSS TQDTVVALHA LSKYGAATFT
RTGKAAQVTI QSSGTFSSKF QVDNNNRLLL QQVSLPELPG EYSMKVTGEG
CVYLQTSLKY NILPEKEEFP FALGVQTLPQ TCDEPKAHTS FQISLSVSYT GSRSASNMAI
VDVKMVSGFI PLKPTVKMLE RSNHVSRTEV SSNHVLIYLD KVSNQTLSLF FTVLQDVPVR
DLKPAIVKVY DYYETDEFAI AEYNAPCSKD LGNA

Wnt Agonists

R-spondin 1 (GenBank: ABC54570.1) Homo sapiens:

(SEQ ID NO: 10)

MRLGLCVVAL VLSWTHLTIS SRGIKGKRQR RISAEGSQAC AKGCELCSEV
NGCLKCSPKL FILLERNDIR QVGVCLPSCP PGYFDARNPD MNKCIKCKIE HCEACFSHNF
CTKCKEGLYL HKGRCYPACP EGSSAANGTM ECSSPAQCEM SEWSPWGPCS
KKQQLCGFRR GSEERTRRVL HAPVGDHAAC SDTKETRRCT VRRVPCPEGQ
KRRKGGQGRR ENANRNLARK ESKEAGAGSR RRKGQQQQQQ QGTVGPLTSA GPA

R-spondin 2 (NCBI Reference Sequence: NP_848660.3) Homo sapiens:

(SEQ ID NO: 11)

MQFRLFSFAL I ILNCMDYSH CQGNRWRRSK RASYVSNPIC KGCLSCSKDN
GCSRCQQKLF FFLRREGMRQ YGECLHSCPS GYYGHRAPDM NRCARCRIEN
CDSCFSKDFC TKCKVGFYLH RGRCFDECPD GFAPLEETME CVEGCEVGHW
SEWGTCSRNN RTCGFKWGLE TRTRQIVKKP VKDTILCPTI AESRRCKMTM
RHCPGGKRTP KAKEKRNKKK KRKLIERAQE QHSVFLATDR ANQ

R-spondin 3 (NCBI Reference Sequence: NP_116173.2) Homo sapiens:

(SEQ ID NO: 12)

MHLRLISWLF I ILNFMEYIG SQNASRGRRQ RRMHPNVSQG CQGGCATCSD YNGCLSCKPR
LFFALERIGM KQIGVCLSSC PSGYYGTRYP DINKCTKCKA DCDTCFNKNF CTKCKSGFYL HLGKCLDNCP EGLEANNHTM ECVSIVHCEV SEWNPWSPCT KKGKTCGFKR GTETRVREI I
QHPSAKGNLC PPTNETRKCT VQRKKCQKGE RGKKGRERKR KKPNKGESKE AIPDSKSLES
SKEIPEQREN KQQQKKRKVQ DKQKSVSVST VH

R-spondin 4 (NCBI Reference Sequence: NP_001025042.2) Homo sapiens: isoform 1 (SEQ ID NO: 13)
MRAPLCLLLL VAHAVDMLAL NRRKKQVGTG LGGNCTGCI I CSEENGCSTC QQRLFLFIRR
EGIRQYGKCL HDCPPGYFGI RGQEVNRCKK CGATCESCFS QDFCIRCKRQ FYLYKGKCLP
TCPPGTLAHQ NTRECQGECE LGPWGGWSPC THNGKTCGSA WGLESRVREA GRAGHEEAAT
CQVLSESRKC PIQRPCPGER SPGQKKGRKD RRPRKDRKLD RRLDVRPRQP GLQP

R-spondin 4 (NCBI Reference Sequence: NP_001035096.1) Homo sapiens: isoform 2 (SEQ ID NO: 14)
MRAPLCLLLL VAHAVDMLAL NRRKKQVGTG LGGNCTGCI I CSEENGCSTC QQRLFLFIRR
EGIRQYGKCL HDCPPGYFGI RGQEVNRCKK CGATCESCFS QDFCIRCKRQ FYLYKGKCLP
TCPPGTLAHQ NTRECQERSP GQKKGRKDRR PRKDRKLDRR LDVRPRQPGL QP

Norrin
norrin precursor [Homo sapiens]
NCBI Reference Sequence: NP_000257.1
(SEQ ID NO: 15)
MRKHVLAASF SMLSLLVIMG DTDSKTDSSF IMDSDPRRCM RHHYVDS I SH PLYKCSSKMV LLARCEGHCS QASRSEPLVS FSTVLKQPFR SSCHCCRPQT SKLKALRLRC SGGMRLTATY RYILSCHCEE CNS

WNT3A [Homo sapiens]
GenBank: BAB61052.1

(SEQ ID NO: 16)
MAPLGYFLLL CSLKQALGSY PIWWSLAVGP QYSSLGSQPI LCASIPGLVP KQLRFCRNYV
EIMPSVAEGI KIGIQECQHQ FRGRRWNCTT VHDSLAIFGP VLDKATRESA FVHAIASAGV
AFAVTRSCAE GTAAICGCSS RHQGSPGKGW KWGGCSEDIE FGGMVSREFA
DARENRPDAR SAMNRHNNEA GRQAIASHMH LKCKCHGLSG SCEVKTCWWS
QPDFRAIGDF LKDKYDSASE MVVEKHRESR GWVETLRPRY TYFKVPTERD
LVYYEASPNF CEPNPETGSF GTRDRTCNVS SHGIDGCDLL CCGRGHNARA
ERRREKCRCV FHWCCYVSCQ ECTRVYDVHT CK

WNT6 [Homo sapiens]
GenBank: AAG45154.1
(SEQ ID NO: 17)
AVGSPLVMDP TSICRKARRL AGRQAELCQA EPEVVAELAR GARLGVRECQ
FQFRFRRWNC SSHSKAFGRI LQQDIRETAF VFAITAAGAS HAVTQACSMG
ELLQCGCQAP RGRAPPRPSG LPGTPGPPGP AGSPEGSAAW EWGGCGDDVD
FGDEKSRLFM DARHKRGRGD IRALVQLHNN EAGRLAVRSH TRTECKCHGL
SGSCALRTCW QKLPPFREVG ARLLERFHGA SRVMGTNDGK ALLPAVRTLK
PPGRADLLYA ADSPDFCAPN RRTGSPGTRG RACNSSAPDL SGCDLLCCGR
GHRQESVQLE ENCLCRFHWC CVVQCHRCRV RKELSLCL

Mitogenic Factors
FGF-2 = bFGF (niProtKB/Swiss-Prot: P09038.3) Homo sapiens:
(SEQ ID NO: 18)
MVGVGGGDVE DVTPRPGGCQ I SGRGARGCN GIPGAAAWEA ALPRRRPRRH
PSVNPRSRAA
GSPRTRGRRT EERPSGSRLG DRGRGRALPG GRLGGRGRGR APERVGGRGR
GRGTAAPRAA
PAARGSRPGP AGTMAAGSIT TLPALPEDGG SGAFPPGHFK DPKRLYCKNG
GFFLRIHPDG
RVDGVREKSD PHIKLQLQAE ERGVVSIKGV CANRYLAMKE DGRLLASKCV
TDECFFFERL
ESNNYNTYRS RKYTSWYVAL KRTGQYKLGS KTGPGQKAIL FLPMSAKS

FGF7 (GenBank: CAG46799.1) Homo sapiens:
(SEQ ID NO: 19)
MHKWILTWIL PTLLYRSCFH IICLVGTISL ACNDMTPEQM ATNVNCSSPE RHTRSYDYME
GGDIRVRRLF CRTQWYLRID KRGKVKGTQE MKNNYNIMEI RTVAVGIVAI KGVESEFYLA

MNKEGKLYAK KECNEDCNFK ELILENHYNT YASAKWTHNG GEMFVALNQK
GIPVRGKKTK KEQKTAHFLP MAIT

FGF10 (GenBank: CAG46489.1) Homo sapiens:
(SEQ ID NO: 20)
MWKWILTHCA SAFPHLPGCC CCFLLLFLV SSVPVTCQAL GQVMVSPEAT
NSSSSSFSSP SSAGRHVRSY NHLQGDVRWR KLFSFTKYFL KIEKNGKVSG
TKKENCPYS I LEITSVEIGV VAVKAINSNY YLAMNKKGKL YGSKEFNNDC KLKERIEENG
YNTYASFNWQ HNGRQMYVAL NGKGAPRRGQ KTRRKNTSAH FLPMVVHS

EGF (GenBank: EAX06257.1) Homo sapiens:
(SEQ ID NO 21)
MLLTLI ILLP VVSKFSFVSL SAPQHWSCPE GTLAGNGNST CVGPAPFLIF SHGNSIFRID
TEGTNYEQLV VDAGVSVIMD FHYNEKRIYW VDLERQLLQR VFLNGSRQER
VCNIEKNVSG MAINWINEEV IWSNQQEGII TVTDMKGNNS HILLSALKYP ANVAVDPVER
FIFWSSEVAG SLYRADLDGV GVKALLETSE KITAVSLDVL DKRLFWIQYN REGSNSLICS
CDYDGGSVHI SKHPTQHNLF AMSLFGDRIF YSTWKMKTIW IANKHTGKDM
VRINLHSSFV PLGELKVVHP LAQPKAEDDT WEPEQKLCKL RKGNCSSTVC
GQDLQSHLCM CAEGYALSRD RKYCEDVNEC AFWNHGCTLG CKNTPGSYYC
TCPVGFVLLP DGKRCHQLVS CPRNVSECSH DCVLTSEGPL CFCPEGSVLE
RDGKTCSGCS SPDNGGCSQL CVPLSPVSWE CDCFPGYDLQ LDEKSCAASG
PQPFLLFANS QDIRHMHFDG TDYGTLLSQQ MGMVYALDHD PVENKIYFAH
TALKWIERAN MDGSQRERLI EEGVDVPEGL AVDWIGRRFY WTDRGKSLIG
RSDLNGKRSK IITKENISQP RGIAVHPMAK RLFWTDTGIN PRIESSSLQG LGRLVIASSD
LIWPSGITID FLTDKLYWCD AKQSVIEMAN LDGSKRRRLT QNDVGHPFAV
AVFEDYVWFS DWAMPSVMRV NKRTGKDRVR LQGSMLKPSS LVVVHPLAKP
GADPCLYQNG GCEHICKKRL GTAWCSCREG FMKASDGKTC LALDGHQLLA
GGEVDLKNQV TPLDILSKTR VSEDNITESQ HMLVAEIMVS DQDDCAPVGC
SMYARCISEG EDATCQCLKG FAGDGKLCSD IDECEMGVPV CPPASSKCIN
TEGGYVCRCS EGYQGDGIHC LDIDECQLGE HSCGENASCT NTEGGYTCMC
AGRLSEPGLI CPDSTPPPHL REDDHHYSVR NSDSECPLSH DGYCLHDGVC
MYIEALDKYA CNCVVGYIGE RCQYRDLKWW ELRHAGHGQQ QKVIVVAVCV
VVLVMLLLLS LWGAHYYRTQ KLLSKNPKNP YEESSRDVRS RRPADTEDGM
SSCPQPWFVV IKEHQDLKNG GQPVAGEDGQ AADGSMQPTS WRQEPQLCGM
GTEQGCWIPV SSDKGSCPQV MERSFHMPSY GTQTLEGGVE KPHSLLSANP
LWQQRALDPP HQMELTQ

TGFa Homo sapiens: protransforming growth factor alpha isoform 1 preproprotein [Homo sapiens] NCBI Reference Sequence: NP_003227.1
(SEQ ID NO: 22)
MVPSAGQLAL FALGIVLAAC QALENSTSPL SADPPVAAAV VSHFNDCPDS
HTQFCFHGTC RFLVQEDKPA CVCHSGYVGA RCEHADLLAV VAASQKKQAI
TALVVVSIVA LAVLIITCVL IHCCQVRKHC EWCRALICRH EKPSALLKGR TACCHSETVV

protransforming growth factor alpha isoform 2 preproprotein [Homo sapiens] NCBI Reference Sequence: NP_001093161.1
(SEQ ID NO: 23)
MVPSAGQLAL FALGIVLAAC QALENSTSPL SDPPVAAAVV SHFNDCPDSH
TQFCFHGTCR FLVQEDKPAC VCHSGYVGAR CEHADLLAVV AASQKKQAIT
ALVVVSIVAL AVLIITCVLI HCCQVRKHCE WCRALICRHE KPSALLKGRT ACCHSETVV

Transforming growth factor alpha [synthetic construct]
GenBank: AAX43291.1
(SEQ ID NO: 24)
MVPLAGQLAL FALGIVLAAC QALENSTSPL SDPPVAAAVV SHFNDCPDSH
TQFCFHGTCR FLVQEDKPAC VCHSGYVGAR CEHADLLAVV AASQKKQAIT
ALVVVSIVAL AVLIITCVLI HCCQVRKHCE WCRALICRHE KPSALLKGRT ACCHSETVVL

TGF alpha containing:
(SEQ ID NO: 25)
VVSHFNDCPD SHTQFCFHGT CRFLVQEDKP ACVCHSGYVG ARCEHA DLLA

BDNF (UniProtKB/Swiss-Prot: P23560.1) Homo sapiens:
(SEQ ID NO 26)
MTILFLTMVI SYFGCMKAAP MKEA IRGQG GLAYPGVRTH GTLESVNGPK AGSRGLTSLA
DTFEHVIEEL LDEDQKVRPN EENNKDADLY TSRVMLSSQV PLEPPLLFLL EEYKNYLDAA
NMSMRVRRHS DPARRGELSV CDSISEWVTA ADKKTAVDMS GGTVTVLEKV
PVSKGQLKQY
FYETKCNPMG YTKEGCRGID KRHWNSQCRT TQSYVRALTM DSKKRIGWRF
IRIDTSCVCT
LTIKRGR

KGF (GenBank: AAB21431.1) Homo sapiens:
(SEQ ID NO: 27)

MHKWILTWIL PTLLYRSCFH IICLVGTISL ACNDMTPEQM ATNVNCSSPE RHTRSYDYME GGDIRVRRLF CRTQWYLRID KRGKVKGTQE MKNNYNIMEI RTVAVGIVAI KGVESEFYLA MNKEGKLYAK KECNEDCNFK ELILENHYNT YASAKWTHNG GEMFVALNQK GIPVRGKKTK KEQKTAHFLP MAIT

Notch Agonist

Jagged-1 (GenBank: ACJ68517.1) *Homo sapiens*:

(SEQ ID NO: 28)

MRSPRTRGRS GRPLSLLLAL LCALRAKVCG ASGQFELEIL SMQNVNGELQ NGNCCGGARN PGDRKCTRDE CDTYFKVCLK EYQSRVTAGG PCSFGSGSTP VIGGNTFNLK ASRGNDRNRI VLPFSFAWPR SYTLLVEAWD SSNDTVQPDS IIEKASHSGM INPSRQWQTL KQNTGVAHFE YQIRVTCDDY YYGFGCNKFC RPRDDFFGHY ACDQNGNKTC MEGWMGPECN RAICRQGCSP KHGSCKLPGD CRCQYGWQGL YCDKCIPHPG CVHGICNEPW QCLCETNWGG QLCDKDLNYC GTHQPCLNGG TCSNTGPDKY QCSCPEGYSG PNCEIAEHAC LSDPCHNRGS CKETSLGFEC ECSPGWTGPT CSTNIDDCSP NNCSHGGTCQ DLVNGFKCVC PPQWTGKTCQ LDANECEAKP CVNAKSCKNL IASYYCDCLP GWMGQNCDIN INDCLGQCQN DASCRDLVNG YRCICPPGYA GDHCERDIDE CASNPCLNGG HCQNEINRFQ CLCPTGFSGN LCQLDIDYCE PNPCQNGAQC YNRASDYFCK CPEDYEGKNC SHLKDHCRTT PCEVIDSCTV AMASNDTPEG VRYISSNVCG PHGKCKSQSG GKFTCDCNKG FTGTYCHENI NDCESNPCRN GGTCIDGVNS YKCICSDGWE GAYCETNIND CSQNPCHNGG TCRDLVNDFY CDCKNGWKGK TCHSRDSQCD EATCNNGGTC YDEGDAFKCM CPGGWEGTTC NIARNSSCLP NPCHNGGTCV VNGESFTCVC KEGWEGPICA QNTNDCSPHP CYNSGTCVDG DNWYRCECAP GFAGPDCRIN INECQSSPCA FGATCVDEIN

GYRCVCPPGH SGAKCQEVSG RPCITMGSVI PDGAKWDDDC NTCQCLNGRI
ACSKVWCGPR
PCLLHKGHSE CPSGQSCIPI LDDQCFVHPC TGVGECRSSS LQPVKTKCTS
DSYYQDNCAN
ITFTFNKEMM SPGLTTEHIC SELRNLNILK NVSAEYSIYI ACEPSPSANN EIHVAISAED
IRDDGNPIKE ITDKIIDLVS KRDGNSSLIA AVAEVRVQRR PLKNRTDFLV PLLSSVLTVA
WICCLVTAFY WCLRKRRKPG SHTHSASEDN TTNNVREQLN QIKNPIEKHG
ANTVPIKDYE
NKNSKMSKIR THNSEVEEDD MDKHQQKARF AKQPAYTLVD REEKPPNGTP
TKHPNWTNKQ
DNRDLESAQS LNRMEYIV

Jagged-1 peptide
(SEQ ID NO: 29)
MRGSHHHHHH GSIEGRSAVT CDDYYYGFGC NKFCRPRDDF FGHYACDQNG
NKTCMEGWMG
PECNRAICRQ GCSPKHGSCK LPGDCRCQYG WQGLYCDKCI PHPGCVHGIC
NEPWQCLCET
NWGGQLCDKD LNYCGTHQPC LNGGTCSNTG PDKYQCSCPE GYSGPNCEI

Jagged-1 peptide
(SEQ ID NO: 30)
CDDYYYGFGCNKFCRPR

Jagged2 [*Homo sapiens*]
GenBank: AAD15562.1
(SEQ ID NO: 31)
MRAQGRGRLP RRLLLLLALW VQAARPMGYF ELQLSALRNV NGELLSGACC
DGDGRTTRAG
GCGHDECDTY VRVCLKEYQA KVTPTGPCSY GHGATPVLGG NSFYLPPAGA
AGDRARARAR
AGGDQDPGLV VIPFQFAWPR SFTLIVEAWD WDNDTTPNEE LLIERVSHAG
MINPEDRWKS
LHFSGHVAHL ELQIRVRCDE NYYSATCNKF CRPRNDFFGH YTCDQYGNKA
CMDGWMGKEC
KEAVCKQGCN LLHGGCTVPG ECRCSYGWQG RFCDECVPYP GCVHGSCVEP
WQCNCETNWG

GLLCDKDLNY CGSHHPCTNG GTCINAEPDQ YRCTCPDGYS GRNCEKAEHA CTSNPCANGG
SCHEVPSGFE CHCPSGWSGP TCALDIDECA SNPCAAGGTC VDQVDGFECI CPEQWVGATC
QLDANECEGK PCLNAFSCKN LIGGYYCDCI PGWKGINCHI NVNDCRGQCQ HGGTCKDLVN
GYQCVCPRGF GGRHCELERD ECASSPCHSG GLCEDLADGF HCHCPQGFSG PLCEVDVDLC
EPSPCRNGAR CYNLEGDYYC ACPDDFGGKN CSVPREPCPG GACRVIDGCG SDAGPGMPGT
AASGVCGPHG RCVSQPGGNF SCICDSGFTG TYCHENIDDC LGQPCRNGGT CIDEVDAFRC
FCPSGWEGEL CDTNPNDCLP DPCHSRGRCY DLVNDFYCAC DDGWKGKTCH SREFQCDAYT
CSNGGTCYDS GDTFRCACPP GWKGSTCAVA KNSSCLPNPC VNGGTCVGSG ASFSCICRDG
WEGRTCTHNT NDCNPLPCYN GGICVDGVNW FRCECAPGFA GPDCRINIDE CQSSPCAYGA
TCVDEINGYR CSCPPGRAGP RCQEVIGFGR SCWSRGTPFP HGSSWVEDCN SCRCLDGRRD
CSKVWCGWKP CLLAGQPEAL SAQCPLGQRC LEKAPGQCLR PPCEAWGECG AEEPPSTPCL
PRSGHLDNNC ARLTLHFNRD HVPQGTTVGA ICSGIRSLPA TRAVARDRLL VLLCDRASSG
ASAVEVAVSF SPARDLPDSS LIQGAAHAIV AAITQRGNSS LLLAVTEVKV ETVVTGGSST
GLLVPVLCGA FSVLWLACVV LCVWWTRKRR KERERSRLPR EESANNQWAP LNPIRNPIER
PGGHKDVLYQ CKNFTPPPRR ADEALPGPAG HAAVREDEED EDLGRGEEDS LEAEKFLSHK
FTKDPGRSPG RPAHWASGPK VDNRAVRSIN EARYAGKE

Delta 1 = delta-like protein 1 (NCBI Reference Sequence: P_005609.3; GenBank: AF196571.1) *Homo sapiens*: SEQ ID NO: 32)

GSRCALALA VLSALLCQVW SSGVFELKLQ EFVNKKGLLG NRNCCRGGAG PPPCACRTFF

RVCLKHYQAS VSPEPPCTYG SAVTPVLGVD SFSLPDGGGA DSAFSNPIRF
PFGFTWPGTF
SLIIEALHTD SPDDLATENP ERLISRLATQ RHLTVGEEWS QDLHSSGRTD LKYSYRFVCD
EHYYGEGCSV FCRPRDDAFG HFTCGERGEK VCNPGWKGPY CTEPICLPGC
DEQHGFCDKP
GECKCRVGWQ GRYCDECIRY PGCLHGTCQQ PWQCNCQEGW GGLFCNQDLN
YCTHHKPCKN
GATCTNTGQG SYTCSCRPGY TGATCELGID ECDPSPCKNG GSCTDLENSY
SCTCPPGFYG
KICELSAMTC ADGPCFNGGR CSDSPDGGYS CRCPVGYSGF NCEKKIDYCS
SSPCSNGAKC
VDLGDAYLCR CQAGFSGRHC DDNVDDCASS PCANGGTCRD GVNDFSCTCP
PGYTGRNCSA
PVSRCEHAPC HNGATCHERG HRYVCECARG YGGPNCQFLL PELPPGPAVV
DLTEKLEGQG
GPFPWVAVCA GVILVLMLLL GCAAVVVCVR LRLQKHRPPA DPCRGETETM
NNLANCQREK
DISVSIIGAT QIKNTNKKAD FHGDHSADKN GFKARYPAVD YNLVQDLKGD DTAVRDAHSK
RDTKCQPQGS SGEEKGTPTT LRGGEASERK RPDSGCSTSK DTKYQSVYVI
SEEKDECVIA
TEV

Delta-4 = delta-like protein 4 precursor [*Homo sapiens*]
NCBI Reference Sequence: NP_061947.1
(SEQ ID NO: 33)
MAAASRSASG WALLLLVALW QQRAAGSGVF QLQLQEFINE RGVLASGRPC
EPGCRTFFRV
CLKHFQAVVS PGPCTFGTVS TPVLGTNSFA VRDDSSGGGR NPLQLPFNFT
WPGTFSLIIE
AWHAPGDDLR PEALPPDALI SKIAIQGSLA VGQNWLLDEQ TSTLTRLRYS YRVICSDNYY
GDNCSRLCKK RNDHFGHYVC QPDGNLSCLP GWTGEYCQQP ICLSGCHEQN
GYCSKPAECL
CRPGWQGRLC NECIPHNGCR HGTCSTPWQC TCDEGWGGLF CDQDLNYCTH
HSPCKNGATC
SNSGQRSYTC TCRPGYTGVD CELELSECDS NPCRNGGSCK DQEDGYHCLC
PPGYYGLHCE

HSTLSCADSP CFNGGSCRER NQGANYACEC PPNFTGSNCE KKVDRCTSNP CANGGQCLNR
GPSRMCRCRP GFTGTYCELH VSDCARNPCA HGGTCHDLEN GLMCTCPAGF SGRRCEVRTS
IDACASSPCF NRATCYTDLS TDTFVCNCPY GFVGSRCEFP VGLPPSFPWV AVSLGVGLAV
LLVLLGMVAV AVRQLRLRRP DDGSREAMNN LSDFQKDNLI PAAQLKNTNQ KKELEVDCGL
DKSNCGKQQN HTLDYNLAPG PLGRGTMPGK FPHSDKSLGE KAPLRLHSEK PECRISAICS
PRDSMYQSVC LISEERNECV IATEV

Delta-like protein 3 isoform 1 precursor [*Homo sapiens*]
NCBI Reference Sequence: NP_058637.1
(SEQ ID NO: 34)
MVSPRMSGLL SQTVILALIF LPQTRPAGVF ELQIHSFGPG PGPGAPRSPC SARLPCRLFF RVCLKPGLSE EAAESPCALG AALSARGPVY TEQPGAPAPD LPLPDGLLQV PFRDAWPGTF
SFIIETWREE LGDQIGGPAW SLLARVAGRR RLAAGGPWAR DIQRAGAWEL RFSYRARCEP
PAVGTACTRL CRPRSAPSRC GPGLRPCAPL EDECEAPLVC RAGCSPEHGF CEQPGECRCL
EGWTGPLCTV PVSTSSCLSP RGPSSATTGC LVPGPGPCDG NPCANGGSCS ETPRSFECTC
PRGFYGLRCE VSGVTCADGP CFNGGLCVGG ADPDSAYICH CPPGFQGSNC EKRVDRCSLQ
PCRNGGLCLD LGHALRCRCR AGFAGPRCEH DLDDCAGRAC ANGGTCVEGG GAHRCSCALG
FGGRDCRERA DPCAARPCAH GGRCYAHFSG LVCACAPGYM GARCEFPVHP DGASALPAAP
PGLRPGDPQR YLLPPALGLL VAAGVAGAAL LLVHVRRRGH SQDAGSRLLA GTPEPSVHAL
PDALNNLRTQ EGSGDGPSSS VDWNRPEDVD PQGIYVISAP SIYAREVATP LFPPLHTGRA
GQRQHLLFPY PSSILSVK

Delta-like protein 3 isoform 2 precursor [*Homo sapiens*]
NCBI Reference Sequence: NP_982353.1
(SEQ ID NO: 35)
MVSPRMSGLL SQTVILALIF LPQTRPAGVF ELQIHSFGPG PGPGAPRSPC SARLPCRLFF
RVCLKPGLSE EAAESPCALG AALSARGPVY TEQPGAPAPD LPLPDGLLQV
PFRDAWPGTF
SFIIETWREE LGDQIGGPAW SLLARVAGRR RLAAGGPWAR DIQRAGAWEL
RFSYRARCEP
PAVGTACTRL CRPRSAPSRC GPGLRPCAPL EDECEAPLVC RAGCSPEHGF
CEQPGECRCL
EGWTGPLCTV PVSTSSCLSP RGPSSATTGC LVPGPGPCDG NPCANGGSCS
ETPRSFECTC
PRGFYGLRCE VSGVTCADGP CFNGGLCVGG ADPDSAYICH CPPGFQGSNC
EKRVDRCSLQ
PCRNGGLCLD LGHALRCRCR AGFAGPRCEH DLDDCAGRAC ANGGTCVEGG
GAHRCSCALG
FGGRDCRERA DPCAARPCAH GGRCYAHFSG LVCACAPGYM GARCEFPVHP
DGASALPAAP
PGLRPGDPQR YLLPPALGLL VAAGVAGAAL LLVHVRRRGH SQDAGSRLLA
GTPEPSVHAL
PDALNNLRTQ EGSGDGPSSS VDWNRPEDVD PQGIYVISAP SIYAREA

d. IBD Gene Sequence/pCD Gene Sequences

Table 3 provides examples of IBD Gene Sequences from certain classes of proteins which are over- or underexpressed in a pediatric Crohn's-derived cells relative to GI stem stem cells from patient matched normal tissue. The gene sequences, such as the coding sequence, mRNA sequence, RNA transcipt or genonmic sequence for Atonal BHLH transcription factor 1 (ATOH1), MUC2, glycoprotein A33 (GPA33), claudin 18 (CLDN18), V-set and immunoglobulin domain containing 1 (VSIG1) or to the genes/proteins identified in Table 3, Figure 1D, Figure 19 are collectively herein the "pCD Gene Sequences".

Table 3

Secretory Proteins	
	CD3(crohn) vs CD3(normal)
Gene Symbol	**Fold change**
MMP7	18.6425
SPINK1	16.2246
MSMB	12.4069
ODAM	10.9916
LCN2	10.1571
TCN1	7.91054
CXCL5	7.19345
SAA1	6.97362
SAA1	6.96736
SERPINB2	6.52824
CXCL2	6.25885
BPIFB1	5.9948
C3	5.69447
KLK7	5.578
CCL20	5.44504
CXCL8	4.37114
F5	4.30107
CCL2	4.2844
KLK6	4.20245
CFB	4.04815
TNFSF15	3.77782
FN1	3.70026
PLA2G10	3.19833
CXCL1	3.10111
SERPINA3	3.0374
COLGALT2	2.96838
VCAN	2.71083
CTGF	2.66206
PRSS1	2.63647

LYPD6B	2.58959
ZG16B	2.56711
DKK1	2.53906
CXCL6	2.47013
PLA2G7	2.46657
RNASE1	2.42255
QPCT	2.35499
EDN1	2.33992
C1GALT1	2.30045
SERPINA4	2.29423
CLU	2.2164
ADAMTS9	2.16422
PRSS23	2.16278
CYP2C18	2.11768
KLK13	2.08632
LEAP2	2.07364
VNN1	2.05267
CCL28	2.00196
COL4A5	1.97145
CTSH	1.96368
LAMA3	1.95609
DYNLT3	1.95361
CHST4	1.9077
PON3	1.89537
SEMA3E	1.88366
FAM107B	1.84363
LIPA	1.81876
HABP2	1.8133
FUCA1	1.80839
SERPINA1	1.80052
SCPEP1	1.79306
GNS	1.78012
TRIM24	1.7754
CYP2C9	1.77381
EPHX3	1.7508
BTD	1.74476

NTF3	1.74126
TTR	1.73938
CYR61	1.73531
DST	1.70862
TIMP3	1.687
KLK11	1.68034
FMOD	1.67048
LIPH	1.66971
PON2	1.66062
EHBP1	1.64904
CTSO	1.64866
CTSL	1.63894
CD24	1.62882
SMOC1	1.62494
NPFF	1.61212
REPS2	1.58951
PDGFC	1.58758
EHF	1.58743
RMDN1	1.56526
IGFBP3	1.56392
B2M	1.56092
HS3ST1	1.53637
EDEM2	1.52645
FSTL4	1.52022
SPINK5	1.50946
B3GALNT2	-1.50138
DDT	-1.51147
TFF3	-1.51359
MRPL24	-1.52982
KDM1A	-1.5446
RNPEP	-1.56895
CES3	-1.57035
CCL24	-1.62428
ITLN2	-1.62539
CLEC3B	-1.62923
BMP6	-1.63385

MGAT4A	-1.66197
CHGB	-1.67242
SERPINE2	-1.67562
GUCA2A	-1.69943
C4BPB	-1.7051
MDK	-1.77843
CCDC108	-1.82922
TPD52	-1.83204
PLA2G12B	-1.86556
ERP27	-1.87949
TDP2	-1.88318
IDNK	-1.89564
INSL4	-2.0073
PI3	-2.14133
KLK1	-2.36205
CNTN1	-2.39373
LPL	-2.41327
CHGA	-2.4657
DPEP1	-2.49415
GAL	-2.51528
KLK12	-2.77691
THBS2	-2.8468
MT1G	-2.89101
FGFBP1	-3.04474
SPON1	-3.44665
CPE	-3.51691
FRZB	-4.18361
TGFBI	-6.42048
SST	-8.87655
FCGBP	-8.90851
PCSK1	-9.40761
PLA2G2A	-9.90775
GCG	-11.6852
RETNLB	-11.8939
REG1A	-14.0148
ITLN1	-18.3747

CLCA1	-22.4519
CEACAM5	-25.0919
ZG16	-35.2239

Cell surface Proteins	
	CD3(crohn) vs CD3(normal)
Gene Symbol	Fold change
VSIG1	10.9645
HLA-DRA	9.92033
CD74	7.27127
LAMP5	6.45444
CLDN18	6.40613
SLC6A14	5.53851
UGT2B15	5.10951
DUOX2	4.55094
DPCR1	4.5179
LRRN1	4.47016
TNFSF10	4.21837
FUT9	4.13202
HLA-DMB	3.82396
TM4SF1	3.54072
GPR87	3.47508
SLC16A4	3.3677
RARRES3	3.19634
FLRT3	3.00196
FREM2	2.98464
GJC1	2.85637
TMC5	2.79718
PVRL3	2.74792
SLC26A9	2.73852
PCDH7	2.72838
STEAP1	2.66904
ITM2A	2.65516
GPRC5B	2.61305
INPP4B	2.44147

SYNE2	2.3778
BAMBI	2.348
KITLG	2.33155
SLC38A11	2.30978
ITGB8	2.30459
ACSL5	2.17559
LPHN3	2.16047
SLC9A2	2.12626
PDZK1IP1	2.08572
TUSC3	2.07371
SLC40A1	2.05513
CFTR	2.03502
HLA-DMA	2.02634
C18orf32	1.98721
TMPRSS2	1.96923
RNF19A	1.95918
GINM1	1.93847
FUT8	1.91954
SLC28A3	1.90876
CYP4F3	1.90556
PHLDB2	1.8956
UST	1.88504
AQP5	1.88295
LAMP2	1.87832
ITGA3	1.85308
YIPF1	1.82643
PRAF2	1.8102
NDRG2	1.79109
SLC7A7	1.7893
ARL6IP5	1.78108
RTP4	1.77623
PTRH2	1.77244
TMEM47	1.75282
TMEM27	1.73888
PEX11B	1.73138
CYP3A5	1.71648

TSPAN1	1.71491
OR4B1	1.71443
CLDN2	1.70997
WRB	1.70886
BCL2L1	1.69254
MYLK	1.68351
GPR22	1.67825
TATDN1	1.67432
SMG8	1.67254
ITGA2	1.67151
CAV2	1.67071
ATP10D	1.6631
DUOXA2	1.65735
RNF128	1.65313
MAOB	1.6479
GJA10	1.64737
PTPRK	1.6442
ITM2B	1.64335
OR7A17	1.64124
TNFRSF10C	1.63969
TNFRSF21	1.63762
SPTLC3	1.63283
ENPP5	1.62833
TGFBR2	1.62244
KCNS3	1.62217
SLCO3A1	1.62112
CASK	1.62014
CD58	1.61747
MYRF	1.61415
MPZL2	1.60863
KCNJ2	1.60648
PLA2G16	1.59827
RNF144B	1.58819
LPAR4	1.58255
SLC1A3	1.58241
CD47	1.58209

ABCC4	1.58178
GJB4	1.58011
SLC1A1	1.57987
STEAP4	1.57841
NBEA	1.57445
DNAJC10	1.5692
FAM174A	1.56841
PCDH11X	1.56408
PROM2	1.56129
GPR75	1.55423
SSBP2	1.55329
SLC4A11	1.55277
LPCAT1	1.55157
SLC9B2	1.5513
ABCC3	1.5384
MAPKAPK3	1.53352
VMP1	1.53084
CD70	1.5287
C5orf15	1.52455
CYP4X1	1.51405
ALCAM	1.50869
PTPLB	1.50557
STEAP2	1.50264
SI	-1.51668
COQ7	-1.5284
DENND1B	-1.53351
MGST3	-1.53673
HILPDA	-1.5446
SLC50A1	-1.5532
SLC17A4	-1.56964
TMEM81	-1.57936
STX2	-1.58659
MAN1A1	-1.59255
IFITM1	-1.59353
EBP	-1.59749
HS6ST2	-1.601

CYC1	-1.6059
SNRPF	-1.6079
SLC7A2	-1.61533
ADORA2B	-1.61844
KCNG3	-1.63455
TMEM200A	-1.64079
CREB3L1	-1.64363
SLC43A1	-1.64561
PARM1	-1.64616
ITM2C	-1.65145
LPCAT2	-1.65299
LPGAT1	-1.6539
MALL	-1.65539
CES2	-1.66016
NEO1	-1.66058
MYEOV	-1.66126
BRI3BP	-1.67212
AGPAT9	-1.6791
ACSS2	-1.67991
TMEM211	-1.69115
ABCB1	-1.6979
TSPAN7	-1.69978
CLDN3	-1.70949
SLC25A44	-1.71404
SLC16A9	-1.72989
RPS15A	-1.73547
DSC2	-1.7499
MBOAT1	-1.75177
INSIG1	-1.75482
MEP1B	-1.7555
HRASLS	-1.7617
FAM3D	-1.78224
CYBRD1	-1.78332
FAR2	-1.78763
P2RX4	-1.82197
TMEM141	-1.83296

SLC7A8	-1.83533
HSD11B2	-1.84618
PLN	-1.84645
TLR4	-1.86452
TMEM171	-1.87842
ST6GALNAC1	-1.88797
PLXDC2	-1.90262
SLC7A5	-1.90363
RNF217	-1.9064
MBOAT2	-1.96393
FOLH1	-1.97633
TRPA1	-1.98184
LBR	-2.01092
PRUNE2	-2.04644
TMED6	-2.07513
GOLT1A	-2.11216
SIDT1	-2.21768
FAM105A	-2.22724
CLRN3	-2.35285
NXPE1	-2.36114
CYP3A4	-2.47472
NOX1	-2.62837
KCNJ3	-2.7739
BTNL3	-2.80196
SLC18A1	-2.83488
DSC3	-3.11227
PRUNE2	-3.12621
NMUR2	-3.21375
FFAR4	-3.72254
TM4SF20	-3.75394
GPA33	-3.79356
CD52	-3.79522
PMP22	-3.86656
ANPEP	-3.89573
CEACAM6	-3.96798
GUCY2C	-7.22138

HEPACAM2	-10.8708

Peptidases	
	CD3(crohn) vs CD3(normal)
Gene Symbol	Fold change
MMP7	18.642
C3	5.694
KLK7	5.578
KLK6	4.202
CFB	4.048
CAPN6	3.802
PRSS1	2.636
ADAMTS9	2.164
PRSS23	2.163
KLK13	2.086
ADAM28	2.025
TMPRSS2	1.969
CTSH	1.964
HABP2	1.813
SCPEP1	1.793
BACE2	1.742
PIGK	1.693
KLK11	1.68
CTSO	1.649
CTSL	1.639
CPM	1.58
PSMB3	-1.536
MYO7B	-1.551
RNPEP	-1.569
MYO1A	-1.582
SPAG5	-1.637
MEP1B	-1.756
CAPN9	-1.847
CASP6	-1.861

FOLH1	-1.976
KLK1	-2.362
DPEP1	-2.494
KLK12	-2.777
PRSS2	-2.974
CPE	-3.517
ANPEP	-3.896
PCSK1	-9.408

Kinases	
	CD3(crohn) vs CD3(normal)
Gene Symbol	Fold change
PLK2	2.887
SGK1	2.113
PRKCA	1.806
NME5	1.719
RIPK2	1.708
ROR1	1.691
MYLK	1.684
TGFBR2	1.622
CASK	1.62
NCK1	1.569
MAPKAPK3	1.534
CDKL5	1.517
NEK2	-1.506
THNSL1	-1.52
PIK3C2B	-1.526
RPS6KA1	-1.583
EPHB2	-1.61
CIT	-1.657
GUK1	-1.675
PRKCZ	-1.698
TRIB3	-1.707
PDK1	-1.803

CKB	-2.097
NDRG1	-4.009
GUCY2C	-7.221

Nucleic acids of the present invention have been identified as differentially expressed in IBD cells, e.g., UC- or CD-derived stem cell lines (relative to the expression levels in normal tissue, e.g., normal colon tissue and/or normal non-colon tissue), such as pCD Gene Sequences. In certain embodiments, the subject nucleic acids are differentially expressed by at least a factor of two, preferably at least a factor of five, even more preferably at least a factor of twenty, still more preferably at least a factor of fifty. In particular, wherein the assay detects a difference in the level of expression of at least a factor of about two, about four, about six, about eight, about ten, about twelve, about fourteen, about sixteen, about eighteen, or about twenty; and more preferably a factor of about twenty-five, about thirty, about thirty-five, about forty, about forty-five, or about fifty.

Genes which are upregulated or downregulated in IBD cells may be targets for diagnostic or therapeutic techniques.

Preferred nucleic acids of the present invention encode a polypeptide comprising at least a portion of a polypeptide encoded by one of the pCD Gene Sequences, or can hybridize to the coding sequences thereof. For example, preferred nucleic acid molecules for use as probes/primers or antisense molecules (i.e., noncoding nucleic acid molecules) can comprise at least about 12, 20, 30, 50, 60, 70, 80, 90, or 100 base pairs in length up to the length of the complete gene. Coding nucleic acid molecules can comprise, for example, from about 50, 60, 70, 80, 90, or 100 base pairs up to the length of the complete gene.

Another aspect of the invention provides a nucleic acid which hybridizes under low, medium, or high stringency conditions to a nucleic acid sequence represented by one of the pCD Gene Sequences, or a sequence complementary thereto. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0×sodium chloride/sodium citrate (SSC) at about 45 C, followed by a wash of 2.0×SSC at 50 C, are known to those skilled in the art or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. 6.3.1-12.3.6 (1989). For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0×SSC at 50 C to a high stringency of about 0.2×SSC at 50 C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 C, to high stringency conditions at about 65 C. Both temperature and salt may be varied, or temperature or salt concentration may be held constant while the other variable is changed. In a preferred embodiment, a nucleic acid of the present invention will hybridize to one of the pCD Gene Sequences, or a sequence complementary thereto, under moderately stringent conditions, for example at

about 2.0×SSC and about 40 C. In a particularly preferred embodiment, a nucleic acid of the present invention will hybridize to one of the pCD Gene Sequences, or a sequence complementary thereto, under high stringency conditions.

In one embodiment, the invention provides nucleic acids which hybridize under low stringency conditions of 6×SSC at room temperature followed by a wash at 2×SSC at room temperature.

In another embodiment, the invention provides nucleic acids which hybridize under high stringency conditions of 2×SSC at 65° C. followed by a wash at 0.2×SSC at 65° C.

Nucleic acids having a sequence that differs from the nucleotide sequences of a pCD Gene Sequence, or a sequence complementary thereto, due to degeneracy in the genetic code, are also within the scope of the invention. Such nucleic acids encode functionally equivalent peptides (i.e., a peptide having equivalent or similar biological activity) but differ in sequence from the sequence shown in the sequence listing due to degeneracy in the genetic code. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC each encode histidine) may result in “silent” mutations which do not affect the amino acid sequence of a polypeptide. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject polypeptides will exist among mammals. One skilled in the art will appreciate that these variations in one or more nucleotides (e.g., up to about 3-5% of the nucleotides) of the nucleic acids encoding polypeptides having an activity of a polypeptide may exist among individuals of a given species due to natural allelic variation.

Also within the scope of the invention are nucleic acids encoding splicing variants of proteins encoded by a nucleic acid of a pCD Gene Sequence, or a sequence complementary thereto, or natural homologs of such proteins. Such homologs can be cloned by hybridization or PCR, as further described herein.

The IBD probes of the present invention can be useful because they provide a method for detecting mutations in wild-type IBD genes of the present invention. Nucleic acid probes which are complementary to a wild-type gene of the present invention and can form mismatches with mutant genes are provided, allowing for detection by enzymatic or chemical cleavage or by shifts in electrophoretic mobility.

Likewise, probes based on the subject sequences can be used to detect the level of transcripts of IBD genes, for use, for example, in prognostic or diagnostic assays. In preferred embodiments, the probe further comprises a label group attached thereto and able to be detected, e.g., the label group is selected from radioisotopes, fluorescent compounds, chemiluminescent compounds, enzymes, and enzyme co-factors.

e. Targeting Expression of IBD Gene Sequence/pCD Gene Sequences

One aspect of the invention relates to the use of the isolated nucleic acid, e.g., from a pCD Gene Sequence that is upregulated in an IBD stem cell population, or a sequence complementary thereto, in antisense therapy or RNA intereference therapy (such as small interfering RNA (siRNA), micro RNA (miRNA) or short-hairpin RNA (shRNA)), a sequence-directed ribozyme or gene inactivating CRISPR RNA (crRNA)

As used herein, antisense therapy refers to administration or in situ generation of oligonucleotide molecules or their derivatives which specifically hybridize (e.g., bind) under cellular conditions with the cellular mRNA and/or genomic DNA, thereby inhibiting transcription and/or translation of that gene. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, antisense therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA. Alternatively, the antisense construct is an oligonucleotide probe which is generated ex vivo and which, when introduced into the cell, causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of a subject nucleic acid. Such oligonucleotide probes are preferably modified oligonucleotides which are resistant to endogenous nucleases, e.g., exonucleases and/or endonucleases, and are therefore stable in vivo. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphorothioate and methylphosphonate analogs of DNA (see also U.S. Pat. No. 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al., BioTechniques 6:958-976 (1988); and Stein et al., Cancer Res. 48:2659-2668 (1988). With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between the –10 and +10 regions of the nucleotide sequence of interest, are preferred.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to mRNA. The antisense oligonucleotides will bind to the mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. In the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled

in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5′ end of the mRNA, e.g., the 5′ untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3′ untranslated sequences of mRNAs have recently been shown to be effective at inhibiting translation of mRNAs as well (Wagner, Nature 372:333 (1994)). Therefore, oligonucleotides complementary to either the 5′ or 3′ untranslated, non-coding regions of a gene could be used in an antisense approach to inhibit translation of endogenous mRNA. Oligonucleotides complementary to the 5′ untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are typically less efficient inhibitors of translation but could also be used in accordance with the invention. Whether designed to hybridize to the 5, 3, or coding region of subject mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably less that about 100 and more preferably less than about 50, 25, 17 or 10 nucleotides in length.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxytriethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

The antisense oligonucleotide can also contain a neutral peptide-like backbone. Such molecules are termed peptide nucleic acid (PNA)-oligomers and are described, e.g., in Perry- O'Keefe et al., Proc. Natl. Acad. Sci. U.S.A. 93:14670 (1996) and in Eglom et al., Nature 365:566 (1993). One advantage of PNA oligomers is their capability to bind to complementary DNA essentially independently from the ionic strength of the medium due to the neutral backbone of the DNA. In yet another embodiment, the antisense oligonucleotide

comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet a further embodiment, the antisense oligonucleotide is an -anomeric oligonucleotide. An -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual -units, the strands run parallel to each other (Gautier et al., Nucl. Acids Res. 15:6625-6641 (1987)). The oligonucleotide is a 2-O-methylribonucleotide (Inoue et al., Nucl. Acids Res. 15:6131-12148 (1987)), or a chimeric RNA-DNA analogue (Inoue et al., FEBS Lett. 215:327-330 (1987)).

The antisense molecules can be delivered to cells which express the target nucleic acid in vivo. A number of methods have been developed for delivering antisense DNA or RNA to cells; e.g., antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (e.g., antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

In another aspect of the invention, ribozyme molecules designed to catalytically cleave target mRNA transcripts corresponding to one or more pCD Gene Sequence can be used to prevent translation of target mRNA and expression of a target protein by the IBD stem cell or its progeny (See, e.g., PCT International Publication WO90/11364; Sarver et al., Science 247:1222-1225 (1990) and U.S. Pat. No. 5,093,246). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy target mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5-UG-3. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, 1988, Nature, 334:585-591. Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5′ end of the target mRNA; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter “Cech-type ribozymes”) such as the one which occurs naturally in Tetrahymena thermophila (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug, et al., Science, 224:574-578 (1984); Zaug and Cech, Science, 231:470-475 (1986); Zaug, et al., Nature, 324:429-433 (1986); published International patent application No. WO88/04300; Been and Cech, Cell, 47:207-216 (1986)). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target

RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in a target pCD Gene Sequence.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (e.g., for improved stability, targeting, etc.) and should be delivered to cells which express the target pCD Gene Sequence in vivo. A preferred method of delivery involves using a DNA construct “encoding” the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous messages and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Antisense RNA, DNA, RNA Interference constructs and ribozyme molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

In other embodiments, the nucleic acid is a a "decoy" nucleic acid which corresponds to a transciptional regulatory sequence and binds to a transcription factor that is involved in upregulated expression of one or more genes in an IBD Stem Cell population. The decoy nucleic acid therefore competes with natural binding target for the binding of the transcription factor and acts an antagonist to reduce the expression of those genes under the transcriptional control of the targeted transcription factor.

f. Therapeutic Nucleic Acids

In some embodiments, a genomic modification (e.g., a deletion of edit of the genome) of an IBD coding sequence is carried out in vivo in a patient using one or more DNA-binding nucleic acids, such as disruption via an RNA-guided endonuclease (RGEN). For example, the disruption can be carried out using clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins. In general, "CRISPR system" refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated ("Cas") genes, including sequences encoding a

Cas gene, a tracr (trans-activating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a "direct repeat" and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a "spacer" in the context of an endogenous CRISPR system), and/or other sequences and transcripts from a CRISPR locus.

The CRISPR/Cas nuclease or CRISPR/Cas nuclease system can include a non-coding RNA molecule (guide) RNA, which sequence-specifically binds to DNA, and a Cas protein (e.g., Cas9), with nuclease functionality (e.g., two nuclease domains). One or more elements of a CRISPR system can derived from a type I, type II, or type III CRISPR system, e.g., derived from a particular organism comprising an endogenous CRISPR system, such as Streptococcus pyogenes.

In some aspects, a Cas nuclease and gRNA (including a fusion of crRNA specific for the target sequence and fixed tracrRNA) are introduced into the patient's cells, i.e., IBD stem cells or cells derived therefrom. In general, target sites at the 5' end of the gRNA target the Cas nuclease to the target site, e.g., the gene, using complementary base pairing. The target site may be selected based on its location immediately 5' of a protospacer adjacent motif (PAM) sequence, such as typically NGG, or NAG. In this respect, the gRNA is targeted to the desired sequence by modifying the first 20 nucleotides of the guide RNA to correspond to the target DNA sequence. In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence. Typically, "target sequence" generally refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between the target sequence and a guide sequence promotes the formation of a CRISPR complex. Full complementarity is not necessarily required, provided there is sufficient complementarity to cause hybridization and promote formation of a CRISPR complex.

The CRISPR system can induce double stranded breaks (DSBs) at the target site, followed by disruptions as discussed herein. In other embodiments, Cas9 variants, deemed "nickases," are used to nick a single strand at the target site. Paired nickases can be used, e.g., to improve specificity, each directed by a pair of different gRNAs targeting sequences such that upon introduction of the nicks simultaneously, a 5' overhang is introduced. In other embodiments, catalytically inactive Cas9 is fused to a heterologous effector.

Typically, in the context of an endogenous CRISPR system, formation of the CRISPR complex (comprising the guide sequence hybridized to the target sequence and complexed with one or more Cas proteins) results in cleavage of one or both strands in or near (e.g. within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from) the target sequence. The tracr sequence, which may comprise or consist of all or a portion of a wild-type tracr sequence (e.g. about or more than about 20, 26, 32, 45, 48, 54, 63, 67, 85, or more

nucleotides of a wild-type tracr sequence), may also form part of the CRISPR complex, such as by hybridization along at least a portion of the tracr sequence to all or a portion of a tracr mate sequence that is operably linked to the guide sequence. The tracr sequence has sufficient complementarity to a tracr mate sequence to hybridize and participate in formation of the CRISPR complex, such as at least 50%, 60%, 70%, 80%, 90%, 95% or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned.

One or more vectors driving expression of one or more elements of the CRISPR system can be introduced into the cell such that expression of the elements of the CRISPR system direct formation of the CRISPR complex at one or more target sites. For example, a Cas enzyme, a guide sequence linked to a tracr-mate sequence, and a tracr sequence could each be operably linked to separate regulatory elements on separate vectors. Alternatively, two or more of the elements expressed from the same or different regulatory elements, may be combined in a single vector, with one or more additional vectors providing any components of the CRISPR system not included in the first vector. The vector may comprise one or more insertion sites, such as a restriction endonuclease recognition sequence (also referred to as a "cloning site"). In some embodiments, one or more insertion sites are located upstream and/or downstream of one or more sequence elements of one or more vectors. When multiple different guide sequences are used, a single expression construct may be used to target CRISPR activity to multiple different, corresponding target sequences within a cell, such as IBD gene sequences such as pCD gene sequences.

A vector may comprise a regulatory element operably linked to an enzyme-coding sequence encoding the CRISPR enzyme, such as a Cas protein. Non-limiting examples of Cas proteins include Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 and Csx12), Cas10, Csy1, Csy2, Csy3, Cse1, Cse2, Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, homologs thereof, or modified versions thereof. These enzymes are known, for example, the amino acid sequence of S. pyogenes Cas9 protein may be found in the SwissProt database under accession number Q99ZW2, incorporated herein by reference.

The CRISPR enzyme can be Cas9 (e.g., from S. pyogenes or S. pneumonia). The CRISPR enzyme can direct cleavage of one or both strands at the location of a target sequence, such as within the target sequence and/or within the complement of the target sequence. The vector can encode a CRISPR enzyme that is mutated with respect to a corresponding wild-type enzyme such that the mutated CRISPR enzyme lacks the ability to cleave one or both strands of a target polynucleotide containing a target sequence. For example, an aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of Cas9

from S. pyogenes converts Cas9 from a nuclease that cleaves both strands to a nickase (cleaves a single strand). In some embodiments, a Cas9 nickase may be used in combination with guide sequence(s), e.g., two guide sequences, which target respectively sense and antisense strands of the DNA target. This combination allows both strands to be nicked and used to induce NHEJ.

In general, a guide sequence is any polynucleotide sequence having sufficient complementarity with a target polynucleotide sequence of an IBD gene sequence to hybridize with the target sequence and direct sequence-specific binding of the CRISPR complex to the target IBD gene sequence. In some embodiments, the degree of complementarity between a guide sequence and its corresponding target IBD gene sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more.

In another embodiment, the gene of interest is useful for inhibiting the expression and/or activity of a nucleic acid or protein of interest. For example, target biomolecule expression and/or activity, such as an RNA coding region, may be reduced or inhibited using inhibitory RNAs. An "RNA coding region" is a nucleic acid that may serve as a template for the synthesis of an RNA molecule, such as an siRNA. "RNA interference (RNAi)" is an evolutionally conserved process whereby the expression or introduction of RNA of a sequence that is identical or highly similar to a target biomarker nucleic acid results in the sequence specific degradation or specific post-transcriptional gene silencing (PTGS) of messenger RNA (mRNA) transcribed from that targeted gene (see, for example, Coburn and Cullen (2002) J. Virol. 76:9225), thereby inhibiting expression of the target biomarker nucleic acid. In one embodiment, the RNA coding region is a DNA sequence. The ability to down-regulate a target IBD gene sequence has many therapeutic and research applications, including identifying the biological functions of particular genes. Moreover, such inhibition may be achieved in screening assays that take advantage of pooling techniques, whereby groups of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, or more, or any number or range in between, of RNA inhibitory agents, either co-expressed from the same vector or more than one vector, are transduced into cells of interest. Suitable inhibitory RNAs include, but are not limited to siRNAs, shRNAs, miRNAs, Piwis, dicer-substrate 27-mer duplexes, single-stranded interfering RNA, and the like. In particular, the combination of RNA inhibitory technology and lentiviruses as a tool for a gene specific knock-down in animal models is well known in the art (see, for example, U.S. Pat. Publ. 2005/0251872; EP Pat. Publ. 2166107; PCT Publs. WO 2004/022722 and 2007/109131; Tiscornia et al. (2003) Proc. Natl. Acad. Sci. U.S.A. 100:1844-1848; Rubinson et al. (2003) Nat. Genet. 33:401-406; and Dann et al. (2006) Proc. Natl. Acad. Sci. U.S.A. 103:11246-11251). As used herein, "inhibition of target biomarker

nucleic acid expression" or "inhibition of marker gene expression" includes any decrease in expression or protein activity or level of the target biomarker nucleic acid or protein encoded by the target biomarker nucleic acid. The decrease may be of at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or more as compared to the expression of a target biomarker nucleic acid or the activity or level of the protein encoded by a target biomarker nucleic acid which has not been targeted by an RNA interfering agent.

siRNAs typically refer to a double-stranded interfering RNA unless otherwise noted. In various embodiments, suitable siRNA molecules include double-stranded ribonucleic acid molecules comprising two nucleotide strands, each strand having about 19 to about 28 nucleotides (i.e. about 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 nucleotides). Thus, the phrase "interfering RNA having a length of 19 to 49 nucleotides" when referring to a double-stranded interfering RNA means that the antisense and sense strands independently have a length of about 19 to about 49 nucleotides, including interfering RNA molecules where the sense and antisense strands are connected by a linker molecule.

In addition to siRNA molecules, other interfering RNA molecules and RNA-like molecules may be used. Examples of other interfering RNA molecules that may to inhibit target biomolecules include, but are not limited to, short hairpin RNAs (shRNAs), single-stranded siRNAs, microRNAs (miRNAs), piwiRNA, dicer-substrate 27-mer duplexes, and variants thereof containing one or more chemically modified nucleotides, one or more non-nucleotides, one or more deoxyribonucleotides, and/or one or more non-phosphodiester linkages. Typically, all RNA or RNA-like molecules that may interact with transcripts RISC complexes and participate in RISC-related changes in gene expression may be referred to as "interfering RNAs" or "interfering RNA molecules." Suitable interfering RNAs may readily be produced based on the well-known nucleotide sequences of target biomolecules. In various embodiments interfering RNAs that inhibit target biomolecules may comprise partially purified RNA, substantially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally-occurring RNA by the addition, deletion, substitution, and/or alteration of one or more nucleotides. Such alterations may include, for example, addition of non-nucleotide material, such as to the end(s) of the interfering RNAs or to one or more internal nucleotides of the interfering RNAs, including modifications that make the interfering RNAs resistant to nuclease digestion. Such alterations result in sequences that are generally at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or more, or 100% identical to the sequence of the target biomolecule. When the gene to be down regulated is in a family of highly conserved genes, the sequence of the duplex region may be chosen with the aid of sequence comparison to target only the desired gene. On the other hand, if there is sufficient identity among a family of homologous genes within an organism,

a duplex region may be designed that would down regulate a plurality of genes simultaneously.

In various embodiments one or both strands of the interfering RNAs may comprise a 3' overhang. As used herein, a "3' overhang" refers to at least one unpaired nucleotide extending from the 3'-end of an RNA strand. Thus in one embodiment, the interfering RNAs comprises at least one 3' overhang of from 1 to about 6 nucleotides (which includes ribonucleotides or deoxynucleotides) in length, from 1 to about 5 nucleotides in length, from 1 to about 4 nucleotides in length, or about 2 to about 4 nucleotides in length. In an illustrative embodiment in which both strands of the interfering RNAs molecule comprise a 3' overhang, wherein the length of the overhangs may be the same or different for each strand. In certain embodiments the 3' overhang is present on both strands of the interfering RNAs and is one, two, or three nucleotides in length. For example, each strand of the interfering RNAs may comprise 3' overhangs of dithymidylic acid ("TT") or diuridylic acid ("uu").

In order to enhance the stability of the interfering RNAs, the 3' overhangs may be also stabilized against degradation. In one embodiment, the overhangs are stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides. In certain embodiments, substitution of pyrimidine nucleotides by modified analogues, e.g., substitution of uridine nucleotides in the 3' overhangs with 2'-deoxythymidine, is tolerated and does not affect the efficiency of RNA interference degradation. In particular, it is believed the absence of a 2' hydroxyl in the 2'-deoxythymidine may significantly enhance the nuclease resistance of the 3' overhang.

Interfering RNAs may be expressed from a vector described herein either as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions. Selection of vectors suitable for expressing interfering RNAs, methods for inserting nucleic acid sequences for expressing the interfering RNAs into the vector, and methods of delivering the recombinant plasmid to the cells of interest are well known in the art (Tuschl (2002) Nat. Biotechnol. 20: 446-448; Brummelkamp et al. (2002) Science 296:550 553; Miyagishi et al. (2002) Nat. Biotechnol. 20:497-500; Paddison et al. (2002) Genes Dev. 16:948-958; Lee et al. (2002) Nat. Biotechnol. 20:500-505; and Paul et al. (2002) Nat. Biotechnol. 20:505-508).

In certain embodiments, the interfering RNAs may be delivered as a small hairpin RNA or short hairpin RNA (shRNA) (see, for example, U.S. Pat. Nos. 8,697,359 and 8,642,569). shRNA is a sequence of RNA that makes a tight hairpin turn that may be used to silence gene expression via RNA interference. In typical embodiments, shRNA uses a vector introduced into cells and utilizes the U6 promoter to ensure that the shRNA is always expressed. This vector is usually passed on to daughter cells, allowing the gene silencing to be inherited. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA,

which is then bound to the RNA-induced silencing complex (RISC). This complex binds to and cleaves mRNAs that match the siRNA that is bound to it.

In certain embodiments, the sense sequence of the shRNA will be from about 19 to about 30, more nucleotides (e.g. about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides) in length, more typically from about 19 to about 22 nucleotides in length, the antisense sequence will be from about 19 to about 30, more typically from 19 to about 22 nucleotides (e.g. about 19, 20, 21 or 22 nucleotides), in length, and the loop region will be from about 3 to about 19 nucleotides (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, and 19 nucleotides) in length. In some embodiments, the sense and antisense sequences are the same length, i.e. the shRNA will form a symmetrical hairpin, but this is not necessarily the case. In some cases, the sense or antisense strand may be shorter than its complementary strand, and an asymmetric hairpin is formed. Further, while in some instances the base pairing between the sense and antisense sequences is exact, this also need not be the case. Thus, some mismatch between the sequences may be tolerated, or even desired, e.g. to decrease the strength of the hydrogen bonding between the two strands. However, in one illustrative embodiment, the sense and antisense sequences are the same length, and the base pairing between the two is exact and does not contain any mismatches. The shRNA molecule may also comprise a 5'-terminal phosphate group that may be chemically modified. In addition, the loop portion of the shRNA molecule may comprise, for example, nucleotides, non-nucleotides, linker molecules, conjugate molecules, etc.

In certain embodiments, the PIWI RNA pathway is used to provide inhibition of target biomolecules. Piwi-interacting RNAs (piRNAs) were identified through association with Piwi proteins in mammalian testes (Aravin et al. (2006); Girard et al. (2006); Grivna et al. (2006); Lau et al. (2006). piRNAs and methods of making and using same to target and degrade nucleic acids are well known in the art (see, for example, U.S. Pat. Publ. 2011-0207625). These RNAs range from 26-30 nucleotides in length and are produced from discrete loci. Generally, genomic regions spanning 50-100 kB in length give rise to abundant piRNAs with profound strand asymmetry. Although the piRNAs themselves are not conserved, even between closely related species, the positions of piRNA loci in related genomes are conserved, with virtually all major piRNA-producing loci having syntenic counterparts in mice, rats and humans (Girard et al. (2006)). The loci and consequently the piRNAs themselves are relatively depleted of repeat and transposon sequences, with only 17% of human piRNAs corresponding to known repetitive elements as compared to a nearly 50% repeat content for the genome as a whole. In certain embodiments, methods are provided for inhibiting such targets in a cell, comprising administering an effective amount of a siRNA/shRNA/piwiRNA to the cell, such that target mRNA is degraded.

In those embodiments for which altered gene expression of an IBD gene sequence, such as a pCD gene sequence, is desired and the therapeutic agent is a nucleic acid, the nucleic acid can be targeted to the IBD stem cells or IBD stem cell derived tissue. "Therapeutic nucleic acids" such as coding sequences for inhibitory domains (dominant negative) versions of the IBD gene sequence, or a CRISPR or other gene editing construct, or antisense or RNA interference construct to inhibit expression of the IBD gene sequence can be delivered by vector or as DNA or mRNA directly to the targeted IBD stem cells or tissue in vivo.

A multitude of clinical studies have illustrated the utility of in vivo gene and nucleic acid transfer into cells using a variety of different delivery systems. Where the therapeutic agent is a polypeptide, or an expressed CRISPR component or an in vivo produced RNA interfence or antisense molecule, a number of expression platforms can be pursued in vivo to which delivery of a therapeutic nucleic acid can be adapted: these include viral vectors, naked DNA and RNA.

An array of physical and chemical nonviral methods have been used to transfer DNA and mRNA to mammalian cells and a substantial number of these have been developed as clinical stage technologies for gene therapy, both ex vivo and in vivo, and are readily adapted for delivery of the therapeutic nucleic acids of the present invention. To illustrate, cationic liposome technology can be employed, which is based on the ability of amphipathic lipids, possessing a positively charged head group and a hydrophobic lipid tail, to bind to negatively charged DNA or RNA and form particles that generally enter cells by endocytosis. Some cationic liposomes also contain a neutral co-lipid, thought to enhance liposome uptake by mammalian cells. See, for example, Felgner et al. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. MNAS 84:7413–7417; San et al. (1983) "Safety and short term toxicity of a novel cationic lipid formulation for human gene therapy" Hum. Gene Ther. 4:781–788; Xu et al. (1996) "Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection" Biochemistry 35,:5616–5623; and Legendre et al. (1992) "Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes" Pharm. Res. 9, 1235-1242.

Similarly, other polycations, such as poly-l-lysine and polyethylene-imine, can be used to deliver therapeutic nucleic acid. These polycations complex with nucleic acids via charge interaction and aid in the condensation of DNA or RNA into nanoparticles, which are then substrates for endosome-mediated uptake. Several of these cationic nucleic acid complex technologies have been developed as potential clinical products, including complexes with plasmid DNA, oligodeoxynucleotides, and various forms of synthetic RNA. Modified (and unmodified or "naked") DNA and RNA have also been shown to mediate successful gene transfer in a number of circumstances and can also be used as systems for

delivery of therapeutic nucleic acid. These include the use of plasmid DNA by direct intramuscular injection, the use of intratumoral injection of plasmid DNA. See, for example, Rodrigo et al. (2012) "De novo automated design of small RNA circuits for engineering synthetic riboregulation in living cells" PNAS 109:15271–15276; Oishi et al. (2005) "Smart polyion complex micelles for targeted intracellular delivery of PEGylated antisense oligonucleotides containing acid-labile linkages" Chembiochem. 6:718–725; Bhatt et al. (2015) "Microbeads mediated oral plasmid DNA delivery using polymethacrylate vectors: an effectual groundwork for colorectal cancer" Drug Deliv. 22:849–861; Ulmer et al. (1994) Protective immunity by intramuscular injection of low doses of influenza virus DNA vaccines" Vaccine 12: 1541–1544; and Heinzerling et al. (2005) "Intratumoral injection of DNA encoding human interleukin 12 into patients with metastatic melanoma: clinical efficacy" Hum. Gene Ther. 16:35–48.

Increased efficiency can also be gained through other techniques, such as in which delivery of the therapeutic nucleic acid is improved by use of chemical carriers—cationic polymers or lipids—or via a physical approach—gene gun delivery or electroporation. See Tranchant et al. (2004) "Physicochemical optimisation of plasmid delivery by cationic lipids" J. Gene Med., 6 (Suppl. 1):S24-S35; and Niidome et al. (2002) "Gene therapy progress and prospects: nonviral vectors" Gene Ther., 9:1647-1652. Electroporation is especially regarded as an interesting technique for nonviral gene delivery. Somiari, et al. (2000) "Theory and in vivo application of electroporative gene delivery" Mol. Ther. 2:178-187; and Jaroszeski et al. (1999) "In vivo gene delivery by electroporation" Adv. Drug Delivery Rev., 35:131-137. With electroporation, pulsed electrical currents are applied to a local tissue area to enhance cell permeability, resulting in gene transfer across the membrane. Research has shown that in vivo gene delivery can be at least 10–100 times more efficient with electroporation than without. See, for example, Aihara et al. (1998) "Gene transfer into muscle by electroporation in vivo" Nat. Biotechnol. 16:867-870; Mir, et al. (1999) "High-efficiency gene transfer into skeletal muscle mediated by electric pulses" PNAS 96:4262-4267; Rizzuto, et al. (1999) "Efficient and regulated erythropoietin production by naked DNA injection and muscle electroporation" PNAS 96: 6417-6422; and Mathiesen (1999) "Electropermeabilization of skeletal muscle enhances gene transfer in vivo" Gene Ther., 6:508-514.

The therapeutic nucleic acids of the present invention can be delivered by a wide range of gene delivery system commonly used for gene therapy including viral, non-viral, or physical. See, for example, Rosenberg et al., Science, 242:1575-1578, 1988, and Wolff et al., Proc. Natl. Acad. Sci. USA 86:9011-9014 (1989). Discussion of methods and compositions for use in gene therapy include Eck et al., in Goodman & Gilman's The Pharmacological Basis of Therapeutics, Ninth Edition, Hardman et al., eds., McGraw-Hill,

New York, (1996), Chapter 5, pp. 77-101; Wilson, Clin. Exp. Immunol. 107 (Suppl. 1):31-32, 1997; Wivel et al., Hematology/Oncology Clinics of North America, Gene Therapy, S. L. Eck, ed., 12(3):483-501, 1998; Romano et al., Stem Cells, 18:19-39, 2000, and the references cited therein. U.S. Pat. No. 6,080,728 also provides a discussion of a wide variety of gene delivery methods and compositions. The routes of delivery include, for example, systemic administration and administration in situ.

g. Polypeptides

The present invention makes available isolated polypeptides which are isolated from, or otherwise substantially free of other cellular proteins, especially other signal transduction factors and/or transcription factors which may normally be associated with the polypeptide. Subject polypeptides of the present invention include polypeptides encoded by pCD Gene Sequences. Polypeptides of the present invention include those proteins which are differentially regulated in IBD tissue, especially colon UC- and CD-derived cell lines (relative to normal cells, e.g., normal colon tissue).

The term “substantially free of other cellular proteins” (also referred to herein as “contaminating proteins”) or “substantially pure or purified preparations” are defined as encompassing preparations of polypeptides having less than about 20% (by dry weight) contaminating protein, and preferably having less than about 5% contaminating protein. Functional forms of the subject polypeptides can be prepared, for the first time, as purified preparations by using a cloned nucleic acid as described herein. Full length proteins or fragments corresponding to one or more particular motifs and/or domains or to arbitrary sizes, for example, at least about 5, 10, 25, 50, 75, or 100 amino acids in length are within the scope of the present invention.

h. Antibodies and other Specific Affinity Binders

The term “specific affinity binder” refers to an antibody as well as to a non-antibody protein scaffold i.e., smaller proteins that are capable of achieving comparable affinity and specificity using molecular structures that can be for example one-fifth to one-tenth the size of full antibodies, and also to nucleic acid aptamers. In some embodiments, the specific affinity binder of the present invention is a non-antibody polypeptide. In some embodiments, the non-antibody polypeptide can include but is not limited to peptibodies, DARPins, avimers, adnectins, anticalins, affibodies, affilins, atrimers, bicyclic peptides, centryins, Cys-knots, Fynomers, Kunitz domains, Obodies, pronectins, Tn3, maxibodies, or other protein structural scaffold, or a combination thereof.

In certain embodiments, the subject invention also provides specific affinity binders, such as antibodies, which selectively bind to a polypetide gene expression product of a pCD

Gene Sequence or other protein that is upregulated in a population of IBD stem cells or its progeny, preferably a protein expressed on the cell surface of the IBD stem cell or its progeny. The binding of the antibody can result in inhibition of the function of the cell, such as proliferation or differentation of IBD stem cells or progeny, cell death, or alteration of the function of the cell in the tissue. As used herein, "selectively binds" or "specifically binds" or "specific binding" in reference to the interaction of an antibody, or antibody fragment thereof, means that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope or target) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope "A", the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled "A" and the antibody, will reduce the amount of labeled A bound to the antibody. In certain embodiments, a binding protein or antibody or antigen-binding fragment thereof that specifically binds to an antigen binds to that antigen with a K_D greater than 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M, 10^{-10} M, 10^{-11} M, 10^{-12} M, 10^{-13} M, 10^{-14} M. In other embodiments, a binding protein or antibody or antigen binding fragment thereof that specifically binds to an antigen binds to that antigen with a K_D between 10^{-6} and 10^{-7M}, 10^{-6} and 10^{-8} M, 10^{-6} and 10^{-9} M, 10^{-6} and 10^{-10} M, 10^{-6} and 10^{-11} M, 10^{-6} and 10^{-12} M, 10^{-6} and 10^{-13} M, 10^{-6} and 10^{-14} M, 10^{-9} and 10^{-10} M, 10^{-9} and 10^{-11} M, 10^{-9} and 10^{-12} M, 10^{-9} and 10^{-13} M, 10^{-9} and 10^{-14} M. In some embodiments, a binding protein or antibody or antigen-binding fragment thereof binds to an epitope, with a K_D 10^{-5} M or less, e.g., 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M, 10^{-10} M, 10^{-11} M, 10^{-12}M, or less. Specific binding can be influenced by, for example, the affinity and avidity of the polypeptide agent and the concentration of polypeptide agent. The person of ordinary skill in the art can determine appropriate conditions under which the polypeptide agents described herein selectively bind the targets using any suitable methods, such as titration of a polypeptide agent in a suitable cell binding assay. In certain embodiments, a binding protein or antibody or antigen-binding fragment thereof is said to "specifically bind" an antigen when it preferentially recognizes its target antigen in a complex mixture of proteins and/or macromolecules. Binding proteins, antibodies or antigen-binding fragments that bind to the same or similar epitopes will likely cross-compete (one prevents the binding or modulating effect of the other). Cross-competition, however, can occur even without epitope overlap, e.g., if epitopes are adjacent in three-dimensional space and/or due to steric hindrance.

In certain embodiments, the antibody is an antibody-drug conjugate, such as an antibody which selectively bind to a polypetide gene expression product of a pCD Gene Sequence or other protein that is upregulated in a population of IBD stem cells or its progeny, which antibody is conjugated to a drug that has a cytotoxic effect, cytostatic effect or epigenetic effect on the IBD stem cell and/or its progeny. “Cytotoxic effect,” in reference to

the effect of an agent on a cell, means killing of the cell. “Cytostatic effect” means an inhibition of cell proliferation. A “cytotoxic agent” means an agent that has a cytotoxic or cytostatic effect on a cell, thereby depleting or inhibiting the growth of, respectively, cells within a cell population.

When used for diagnostic or in vivo imaging purposes, the antibody can be conugated to a detectable label, such as such as enzymes, DNA segments, fluorescent compounds, imaging agents, dyes and the like.

Antibodies suitable for use in accordance with the present compositions and methods are typically monoclonal and can include, for example, chimeric (e.g., having a human constant region and mouse variable region), humanized, or human antibodies; single chain antibodies; or the like. The immunoglobulin molecules can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule.

In certain embodiments, the antibody is an antigen-binding antibody fragment such as, for example, a Fab, a F(ab′), a F(ab′)2, a Fd chain, a single-chain Fv (scFv), a single-chain antibody, a disulfide-linked Fv (sdFv), a fragment comprising either a VL or VH domain, or fragments produced by a Fab expression library, or an antigen-binding fragments of any of the above antibodies described supra. Antigen-binding antibody fragments, including single-chain antibodies, can comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, CH3 and CL domains. Also, antigen-binding fragments can comprise any combination of variable region(s) with a hinge region, CH1, CH2, CH3 and CL domains. Typically, the antibodies are human, rodent (e.g., mouse and rat), donkey, sheep, rabbit, goat, guinea pig, camelid, horse, or chicken. As used herein, “human” antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries, from human B cells, or from animals transgenic for one or more human immunoglobulin, as described infra and, for example in U.S. Pat. Nos. 5,939,598 and 6,111,166.

The antibodies maybe monospecific, bispecific, trispecific, or of greater multispecificity. Multispecific antibodies maybe specific for different epitopes of the same protein or may be specific for two different proteins. (See, e.g., PCT publications WO 93/17715; WO 92/08802; WO 91/00360; and WO 92/05793; Tutt et al., 1991, J Immunol 147:60-69; U.S. Pat. Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920; and 5,601,819; Kostelny et al., 1992, J Immunol 148:1547-1553.) Multispecific antibodies, including bispecific and trispecific antibodies, useful for practicing the methods described include herein are antibodies that immunospecifically bind to both an antigen on a protein selectively expressed by the IBD stem cell or its progeny, such as may be encoded by a pCD Gene

Sequence and a second cell surface receptor or receptor complex, such as an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytoliine receptor, a chemokine receptor, a major histocompatibility protein, a lectin (C-type, S-type, or I-type), or a complement control protein. In a typical embodiment, the binding of the portion of the multispecific antibody to the second cell surface molecule or receptor complex enhances the cytotoxic or cytostatic effect of an antibody-drug conjugate.

In certain specific embodiments, the antibody is agonistic, non-agonistic or antagonistic with respective to the function of the protein to which is binds.

(i) Selective Delivery of Drug Conjugates

In certain embodiments, the IBD targeting antibody or other IBD selective affinity binder has a drug, toxin or other pharmacologically active moiety (collectively a "therapeutic agent" attached thereto, either covalently or non-covalently, such that the drug conjugate is preferentially released when the specific affinity binder is assocated with tissue expressing the antigen to which the specific affinity binder binds, such as an antigen on the surface of an IBD stem cells or tissue derived therefrom.

As used herein, the term "therapeutic agent" refers to a substance that may be used in the cure, mitigation, treatment, or prevention of an inflammatory bowel disease in a human or another animal. Such therapeutic agents include substances recognized in the official United States Pharmacopeia, official Homeopathic Pharmacopeia of the United States, official National Formulary, or any supplement thereof, and include but are not limited to small molecules, nucleotides, oligopeptides, polypeptides, etc.

Therapeutic agents that may be attached to specific affinity binder polypeptides for selective killing of IBD stem cells or IBD stem cell derived tissues include, but are not limited to, cytotoxic agents, anti-metabolites, alkylating agents, antibiotics, growth factor, cytokines, anti-angiogenic agents, anti-mitotic agents, toxins, apoptotic agents or the like, such as DNA alkylating agents, topoisomerase inhibitors, endoplasmic reticulum stress inducing agents, platinum compounds, antimetabolites, vincalkaloids, taxanes, epothilones, enzyme inhibitors, receptor antagonists, therapeutic antibodies, tyrosine kinase inhibitors, radiosensitizers, and chemotherapeutic combination therapies, such as illustrations.

Non-limiting examples of DNA alkylating agents are nitrogen mustards, such as Mechlorethamine, Cyclophosphamide (Ifosfamide, Trofosfamide), Chlorambucil (Melphalan, Prednimustine), Bendamustine, Uramustine and Estramustine; nitrosoureas, such as Carmustine (BCNU), Lomustine (Semustine), Fotemustine, Nimustine, Ranimustine and Streptozocin; alkyl sulfonates, such as Busulfan (Mannosulfan, Treosulfan); Aziridines, such as Carboquone, ThioTEPA, Triaziquone, Triethylenemelamine; Hydrazines (Procarbazine); Triazenes such as Dacarbazine and Temozolomide; Altretamine and Mitobronitol.

Non-limiting examples of Topoisomerase I inhibitors include Campothecin derivatives including CPT-11 (irinotecan), SN-38, APC, NPC, campothecin, topotecan, exatecan mesylate, 9-nitrocamptothecin, 9-aminocamptothecin, lurtotecan, rubitecan, silatecan, gimatecan, diflomotecan, extatecan, BN-80927, DX-8951f, and MAG-CPT as described in Pommier Y. (2006) Nat. Rev. Cancer 6(10):789-802 and U.S. Patent Publication No. 200510250854; Protoberberine alkaloids and derivatives thereof including berberrubine and coralyne as described in Li et al. (2000) Biochemistry 39(24):7107-7116 and Gatto et al. (1996) Cancer Res. 15(12):2795-2800; Phenanthroline derivatives including Benzo[i]phenanthridine, Nitidine, and fagaronine as described in Makhey et al. (2003) Bioorg. Med. Chem. 11 (8): 1809-1820; Terbenzimidazole and derivatives thereof as described in Xu (1998) Biochemistry 37(10):3558-3566; and Anthracycline derivatives including Doxorubicin, Daunorubicin, and Mitoxantrone as described in Foglesong et al. (1992) Cancer Chemother. Pharmacol. 30(2):123-]25, Crow et al. (1994) J. Med. Chem. 37(19):31913194, and Crespi et al. (1986) Biochem. Biophys. Res. Commun. 136(2):521-8. Topoisomerase II inhibitors include, but are not limited to Etoposide and Teniposide. Dual topoisomerase I and II inhibitors include, but are not limited to, Saintopin and other Naphthecenediones, DACA and other Acridine-4-Carboxamindes, Intoplicine and other Benzopyridoindoles, TAS-103 and other 7H-indeno[2,1-c]Quinoline-7-ones, Pyrazoloacridine, XR 11576 and other Benzophenazines, XR 5944 and other Dimeric compounds, 7-oxo-7H-dibenz[f,ij]Isoquinolines and 7-oxo-7H-benzo[e]Perimidines, and Anthracenyl-amino Acid Conjugates as described in Denny and Baguley (2003) Curr. Top. Med. Chem. 3(3):339-353. Some agents inhibit Topoisomerase II and have DNA intercalation activity such as, but not limited to, Anthracyclines (Aclarubicin, Daunorubicin, Doxorubicin, Epirubicin, Idarubicin, Amrubicin, Pirarubicin, Valrubicin, Zorubicin) and Antracenediones (Mitoxantrone and Pixantrone).

Examples of endoplasmic reticulum stress inducing agents include, but are not limited to, dimethyl-celecoxib (DMC), nelfinavir, celecoxib, and boron radiosensitizers (i.e. velcade (Bortezomib)).

Non-limiting examples of platinum-based compound include Carboplatin, Cisplatin, Nedaplatin, Oxaliplatin, Triplatin tetranitrate, Satraplatin, Aroplatin, Lobaplatin, and JM-216. (see McKeage et al. (1997) J. Clin. Oncol. 201:1232-1237 and in general, CHEMOTHERAPY FOR GYNECOLOGICAL NEOPLASM, CURRENT THERAPY AND NOVEL APPROACHES, in the Series Basic and Clinical Oncology, Angioli et al. Eds., 2004).

Non-limiting examples of antimetabolite agents include Folic acid based, i.e. dihydrofolate reductase inhibitors, such as Aminopterin, Methotrexate and Pemetrexed; thymidylate synthase inhibitors, such as Raltitrexed, Pemetrexed; Purine based, i.e. an

adenosine deaminase inhibitor, such as Pentostatin, a thiopurine, such as Thioguanine and Mercaptopurine, a halogenated/ribonucleotide reductase inhibitor, such as Cladribine, Clofarabine, Fludarabine, or a guanine/guanosine: thiopurine, such as Thioguanine; or Pyrimidine based, i.e. cytosine/cytidine: hypomethylating agent, such as Azacitidine and Decitabine, a DNA polymerase inhibitor, such as Cytarabine, a ribonucleotide reductase inhibitor, such as Gemcitabine, or a thymine/thymidine: thymidylate synthase inhibitor, such as a Fluorouracil (5-FU). Equivalents to 5-FU include prodrugs, analogs and derivative thereof such as 5′-deoxy-5-fluorouridine (doxifluoroidine), 1-tetrahydrofuranyl-5-fluorouracil (ftorafur), Capecitabine (Xeloda), S-I (MBMS-247616, consisting of tegafur and two modulators, a 5-chloro-2,4-dihydroxypyridine and potassium oxonate), ralititrexed (tomudex), no latrexed (Thymitaq, AG337), LY231514 and ZD9331, as described for example in Papamicheal (1999) The Oncologist 4:478-487.

Examples of vincalkaloids, include, but are not limited to Vinblastine, Vincristine, Vinflunine, Vindesine and Vinorelbine.

Examples of taxanes include, but are not limited to docetaxel, Larotaxel, Ortataxel, Paclitaxel and Tesetaxel. An example of an epothilone is iabepilone.

Examples of enzyme inhibitors include, but are not limited to farnesyltransferase inhibitors (Tipifamib); CDK inhibitor (Alvocidib, Seliciclib); proteasome inhibitor (Bortezomib); phosphodiesterase inhibitor (Anagrelide; rolipram); IMP dehydrogenase inhibitor (Tiazofurine); and lipoxygenase inhibitor (Masoprocol). Examples of receptor antagonists include, but are not limited to ERA (Atrasentan); retinoid X receptor (Bexarotene); and a sex steroid (Testolactone).

Examples of therapeutic antibodies include, but are not limited to anti-HER1/EGFR (Cetuximab, Panitumumab); Anti-HER2/neu (erbB2) receptor (Trastuzumab); Anti-EpCAM (Catumaxomab, Edrecolomab) Anti-VEGF-A (Bevacizumab); Anti-CD20 (Rituximab, Tositumomab, Ibritumomab); Anti-CD52 (Alemtuzumab); and Anti-CD33 (Gemtuzumab). U.S. Pat. Nos. 5,776,427 and 7,601,355.

Examples of tyrosine kinase inhibitors include, but are not limited to inhibitors to ErbB: HER1/EGFR (Erlotinib, Gefitinib, Lapatinib, Vandetanib, Sunitinib, Neratinib); HER2/neu (Lapatinib, Neratinib); RTK class III: C-kit (Axitinib, Sunitinib, Sorafenib), FLT3 (Lestaurtinib), PDGFR (Axitinib, Sunitinib, Sorafenib); and VEGFR (Vandetanib, Semaxanib, Cediranib, Axitinib, Sorafenib); bcr-abl (Imatinib, Nilotinib, Dasatinib); Src (Bosutinib) and Janus kinase 2 (Lestaurtinib).

Chemotherapeutic agents that can be attached to the present specific affinity binder polypeptides may also include amsacrine, Trabectedin, retinoids (Alitretinoin, Tretinoin), Arsenic trioxide, asparagine depleter Asparaginase/Pegaspargase), Celecoxib,

Demecolcine, Elesclomol, Elsamitrucin, Etoglucid, Lonidamine, Lucanthone, Mitoguazone, Mitotane, Oblimersen, Temsirolimus, and Vorinostat.

Examples of specific therapeutic agents that can be linked, ligated, or associated with the specific affinity binder polypeptides of the invention are flomoxef; fortimicin(s); gentamicin(s); glucosulfone solasulfone; gramicidin S; gramicidin(s); grepafloxacin; guamecycline; hetacillin; isepamicin; josamycin; kanamycin(s); flomoxef; fortimicin(s); gentamicin(s); glucosulfone solasulfone; gramicidin S; gramicidin(s); grepafloxacin; guamecycline; hetacillin; isepamicin; josamycin; kanamycin(s); bacitracin; bambermycin(s); biapenem; brodimoprim; butirosin; capreomycin; carbenicillin; carbomycin; carumonam; cefadroxil; cefamandole; cefatrizine; cefbuperazone; cefclidin; cefdinir; cefditoren; cefepime; cefetamet; cefixime; cefinenoxime; cefininox; cladribine; apalcillin; apicycline; apramycin; arbekacin; aspoxicillin; azidamfenicol; aztreonam; cefodizime; cefonicid; cefoperazone; ceforamide; cefotaxime; cefotetan; cefotiam; cefozopran; cefpimizole; cefpiramide; cefpirome; cefprozil; cefroxadine; cefteram; ceftibuten; cefuzonam; cephalexin; cephaloglycin; cephalosporin C; cephradine; chloramphenicol; chlortetracycline; clinafloxacin; clindamycin; clomocycline; colistin; cyclacillin; dapsone; demeclocycline; diathymosulfone; dibekacin; dihydrostreptomycin; 6-mercaptopurine; thioguanine; capecitabine; docetaxel; etoposide; gemcitabine; topotecan; vinorelbine; vincristine; vinblastine; teniposide; melphalan; methotrexate; 2-p-sulfanilyanilinoethanol; 4,4′-sulfinyldianiline; 4-sulfanilamidosalicylic acid; butorphanol; nalbuphine. streptozocin; doxorubicin; daunorubicin; plicamycin; idarubicin; mitomycin C; pentostatin; mitoxantrone; cytarabine; fludarabine phosphate; butorphanol; nalbuphine. streptozocin; doxorubicin; daunorubicin; plicamycin; idarubicin; mitomycin C; pentostatin; mitoxantrone; cytarabine; fludarabine phosphate; acediasulfone; acetosulfone; amikacin; amphotericin B; ampicillin; atorvastatin; enalapril; ranitidine; ciprofloxacin; pravastatin; clarithromycin; cyclosporin; famotidine; leuprolide; acyclovir; paclitaxel; azithromycin; lamivudine; budesonide; albuterol; indinavir; metformin; alendronate; nizatidine; zidovudine; carboplatin; metoprolol; amoxicillin; diclofenac; lisinopril; ceftriaxone; captopril; salmeterol; xinafoate; imipenem; cilastatin; benazepril; cefaclor; ceftazidime; morphine; dopamine; bialamicol; fluvastatin; phenamidine; podophyllinic acid 2-ethylhydrazine; acriflavine; chloroazodin; arsphenamine; amicarbilide; aminoquinuride; quinapril; oxymorphone; buprenorphine; floxuridine; dirithromycin; doxycycline; enoxacin; enviomycin; epicillin; erythromycin; leucomycin(s); lincomycin; lomefloxacin; lucensomycin; lymecycline; meclocycline; meropenem; methacycline; micronomicin; midecamycin(s); minocycline; moxalactam; mupirocin; nadifloxacin; natamycin; neomycin; netilmicin; norfloxacin; oleandomycin; oxytetracycline; p-sulfanilylbenzylamine; panipenem; paromomycin; pazufloxacin; penicillin N; pipacycline; pipemidic acid; polymyxin; primycin; quinacillin; ribostamycin; rifamide; rifampin; rifamycin

SV; rifapentine; rifaximin; ristocetin; ritipenem; rokitamycin; rolitetracycline; rosaramycin; roxithromycin; salazosulfadimidine; sancycline; sisomicin; sparfloxacin; spectinomycin; spiramycin; streptomycin; succisulfone; sulfachrysoidine; sulfaloxic acid; sulfamidochrysoidine; sulfanilic acid; sulfoxone; teicoplanin; temafloxacin; temocillin; tetroxoprim; thiamphenicol; thiazolsulfone; thiostrepton; ticarcillin; tigemonam; tobramycin; tosufloxacin; trimethoprim; trospectomycin; trovafloxacin; tuberactinomycin; vancomycin; azaserine; candicidin(s); chlorphenesin; dermostatin(s); filipin; fungichromin; mepartricin; nystatin; oligomycin(s); perimycin A; tubercidin; 6-azauridine; 6-diazo-5-oxo-L-norleucine; aclacinomycin(s); ancitabine; anthramycin; azacitadine; azaserine; bleomycin(s); ethyl biscoumacetate; ethylidene dicoumarol; iloprost; lamifiban; taprostene; tioclomarol; tirofiban; amiprilose; bucillamine; gusperimus; gentisic acid; glucamethacin; glycol salicylate; meclofenamic acid; mefenamic acid; mesalamine; niflumic acid; olsalazine; oxaceprol; S-enosylmethionine; salicylic acid; salsalate; sulfasalazine; tolfenamic acid; carubicin; carzinophillin A; chlorozotocin; chromomycin(s); denopterin; doxifluridine; edatrexate; eflornithine; elliptinium; enocitabine; epirubicin; mannomustine; menogaril; mitobronitol; mitolactol; mopidamol; mycophenolic acid; nogalamycin; olivomycin(s); peplomycin; pirarubicin; piritrexim; prednimustine; procarbazine; pteropterin; puromycin; ranimustine; streptonigrin; thiamiprine; mycophenolic acid; procodazole; romurtide; sirolimus (rapamycin); tacrolimus; butethamine; fenalcomine; hydroxytetracaine; naepaine; orthocaine; piridocaine; salicyl alcohol; 3-amino-4-hydroxybutyric acid; aceclofenac; alminoprofen; amfenac; bromfenac; bromosaligenin; bumadizon; carprofen; diclofenac; diflunisal; ditazol; enfenamic acid; etodolac; etofenamate; fendosal; fepradinol; flufenamic acid; Tomudex (N-[[5-[[(1,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]methylamino]-2-thienyl]carbonyl]-L-glutamic acid), trimetrexate, tubercidin, ubenimex, vindesine, zorubicin; argatroban; coumetarol or dicoumarol.

In certain embodiments, the IBD targeting antibody or other selective affinity binder includes a conjugated cytotoxic factor such as diptheria toxin, Pseudomonas aeruginosa exotoxin A chain, ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins and compounds (e.g., fatty acids), dianthin proteins, Phytoiacca americana proteins PAPI, PAPII, and PAP-S, momordica charantia inhibitor, curcin, crotin, saponaria officinalis inhibitor, mitogellin, restrictocin, phenomycin, and enomycin.

In certain embodiments, the IBD targeting antibody or other selective affinity binder includes a conjugated anti-inflammatory or immunosuppressive agent that is released in the area of the targeted IBD stem cell or IBD stem cell derived tissue.

In certain embodiments, the IBD targeting antibody or other selective affinity binder includes a conjugated epigenetic agent that induces one or more epigenetic changes to the targeted IBD stem cell or IBD stem cell derived tissue, such as resulting in cell death,

inhibition of proliferation, inhibition of differentiation, and/or altered tissue fate upon differentiation of the targeted IBD stem cell such that the resulting tissue is either normal or at least results in reduced symptoms or inflammation during the course of the disease.

Any method known in the art for conjugating to antibodies and other proteins may be employed in generating the conjugates of the present invention, including those methods described by Hunter, et al., (1962) Nature 144:945; David, et al., (1974) Biochemistry 13:1014; Pain, et al., (1981) J. Immunol. Meth. 40:219; and Nygren, J., (1982) Histochem. and Cytochem. 30:407. Methods for conjugating peptide, polypeptide and organic and inorganic moieties to antibodies and other proteins are conventional and very well known in the art and readily adapted for generating those versions of the subject IBD targeting antibody or other IBD selective affinity binder.

Where the conjugated moiety is a peptide or polypeptide, that moiety can be chemically cross-linked to the specific affinity binder, or can be included as part of a fusion protein with the specific affinity binder polypeptide. And illustrative example would be a diptheria toxin-antibody fusion protein. In the case of non-peptide entities, the addition to the specific affinity binder polypeptide will generally be by way of chemical conjugation to the specific affinity binder polypeptide – such as through a functional group on an amino acid side chain or the carboxyl group at the C-terminal or amino group at the N-terminal end of the polypeptide. In certain embodiment, whether as a fusion protein or chemically cross-linked moiety, the conjugated moiety will include one or more sites that can be cleaved by an enzyme or are otherwise sensitive to an environmental condition (such as pH) that permits the conjugated moiety to be released from the specific affinity binder polypeptide, such as in the tumour or other diseased tissue (or tissue to be protected if the conjugated moiety functions to protect healthy tissue).

i. Diagnostics

The subject invention further provides a method of determining whether a cell sample obtained from a subject possesses an abnormal amount of marker polypeptide which comprises (a) obtaining a cell sample from the subject, (b) quantitatively determining the amount of the marker polypeptide in the sample so obtained, and (c) comparing the amount of the marker polypeptide so determined with a known standard, so as to thereby determine whether the cell sample obtained from the subject possesses an abnormal amount of the marker polypeptide. Such marker polypeptides may be detected by immunohistochemical assays, dot-blot assays, ELISA and the like.

Immunoassays are commonly used to quantitate the levels of proteins in cell samples, and many other immunoassay techniques are known in the art. The invention is not limited to a particular assay procedure, and therefore is intended to include both

homogeneous and heterogeneous procedures. Exemplary immunoassays which can be conducted according to the invention include fluorescence polarization immunoassay (FPIA), fluorescence immunoassay (FIA), enzyme immunoassay (EIA), nephelometric inhibition immunoassay (NIA), enzyme linked immunosorbent assay (ELISA), and radioimmunoassay (RIA). An indicator moiety, or label group, can be attached to the subject antibodies and is selected so as to meet the needs of various uses of the method which are often dictated by the availability of assay equipment and compatible immunoassay procedures. General techniques to be used in performing the various immunoassays noted above are known to those of ordinary skill in the art.

In another embodiment, the level of the encoded product, i.e., the product encoded by an IBD gene (such as an pCD Gene Sequence) or a sequence complementary thereto, in a biological fluid (e.g., blood or urine) of a patient may be determined as a way of monitoring the level of expression of the marker nucleic acid sequence in cells of that patient. Such a method would include the steps of obtaining a sample of a biological fluid from the patient, contacting the sample (or proteins from the sample) with an antibody specific for a encoded marker polypeptide, and determining the amount of immune complex formation by the antibody, with the amount of immune complex formation being indicative of the level of the marker encoded product in the sample. This determination is particularly instructive when compared to the amount of immune complex formation by the same antibody in a control sample taken from a normal individual or in one or more samples previously or subsequently obtained from the same person.

As set out above, one aspect of the present invention relates to diagnostic assays for determining, in the context of cells isolated from a patient, if the level of a marker polypeptide is significantly reduced in the sample cells. The term “significantly reduced” refers to a cell phenotype wherein the cell possesses a reduced cellular amount of the marker polypeptide relative to a normal cell of similar tissue origin. For example, a cell may have less than about 50%, 25%, 10%, or 5% of the marker polypeptide that a normal control cell. In particular, the assay evaluates the level of marker polypeptide in the test cells, and, preferably, compares the measured level with marker polypeptide detected in at least one control cell, e.g., a normal cell and/or a transformed cell of known phenotype.

Of particular importance to the subject invention is the ability to quantitate the level of marker polypeptide as determined by the number of cells associated with a normal or abnormal marker polypeptide level. The number of cells with a particular marker polypeptide phenotype may then be correlated with patient prognosis. In one embodiment of the invention, the marker polypeptide phenotype of the lesion is determined as a percentage of cells in a biopsy which are found to have abnormally high/low levels of the marker

polypeptide. Such expression may be detected by immunohistochemical assays, dot-blot assays, ELISA and the like.

Where tissue samples are employed, immunohistochemical staining may be used to determine the number of cells having the marker polypeptide phenotype. For such staining, a multiblock of tissue is taken from the biopsy or other tissue sample and subjected to proteolytic hydrolysis, employing such agents as protease K or pepsin. In certain embodiments, it may be desirable to isolate a nuclear fraction from the sample cells and detect the level of the marker polypeptide in the nuclear fraction.

The tissue samples are fixed by treatment with a reagent such as formalin, glutaraldehyde, methanol, or the like. The samples are then incubated with an antibody, preferably a monoclonal antibody, with binding specificity for the marker polypeptides. This antibody may be conjugated to a label for subsequent detection of binding. Samples are incubated for a time sufficient for formation of the immuno-complexes. Binding of the antibody is then detected by virtue of a label conjugated to this antibody. Where the antibody is unlabeled, a second labeled antibody may be employed, e.g., which is specific for the isotype of the anti-marker polypeptide antibody. Examples of labels which may be employed include radionuclides, fluorescers, chemiluminescers, enzymes and the like.

Where enzymes are employed, the substrate for the enzyme may be added to the samples to provide a colored or fluorescent product. Examples of suitable enzymes for use in conjugates include horseradish peroxidase, alkaline phosphatase, malate dehydrogenase and the like. Where not commercially available, such antibody-enzyme conjugates are readily produced by techniques known to those skilled in the art.

In one embodiment, the assay is performed as a dot blot assay. The dot blot assay finds particular application where tissue samples are employed as it allows determination of the average amount of the marker polypeptide associated with a single cell by correlating the amount of marker polypeptide in a cell-free extract produced from a predetermined number of cells.

In one embodiment, the present invention also provides a method wherein nucleic acid probes are immobilized on a DNA chip in an organized array. Oligonucleotides can be bound to a solid support by a variety of processes, including lithography. For example a chip can hold up to 250,000 oligonucleotides (GeneChip, Affymetrix). These nucleic acid probes comprise a nucleotide sequence at least about 12 nucleotides in length, preferably at least about 15 nucleotides, more preferably at least about 25 nucleotides, and most preferably at least about 40 nucleotides, and up to all or nearly all of a sequence which is complementary to a portion of the coding sequence of one or more marker nucleic acid sequence for pCD Gene Sequences.

The method includes obtaining a biopsy, which is optionally fractionated by cryostat sectioning to enrich tumor cells to about 80% of the total cell population. The DNA or RNA is then extracted, amplified, and analyzed with a DNA chip to determine the presence of absence of the marker nucleic acid sequences.

In one embodiment, the nucleic acid probes are spotted onto a substrate in a two-dimensional matrix or array. Samples of nucleic acids can be labeled and then hybridized to the probes. Double-stranded nucleic acids, comprising the labeled sample nucleic acids bound to probe nucleic acids, can be detected once the unbound portion of the sample is washed away.

The probe nucleic acids can be spotted on substrates including glass, nitrocellulose, etc. The probes can be bound to the substrate by either covalent bonds or by non-specific interactions, such as hydrophobic interactions. The sample nucleic acids can be labeled using radioactive labels, fluorophores, chromophores, etc.

Techniques for constructing arrays and methods of using these arrays are described in EP No. 0 799 897; PCT No. WO 97/29212; PCT No. WO 97/27317; EP No. 0 785 280; PCT No. WO 97/02357; U.S. Pat. No. 5,593,839; U.S. Pat. No. 5,578,832; EP No. 0 728 520; U.S. Pat. No. 5,599,695; EP No. 0 721 016; U.S. Pat. No. 5,556,752; PCT No. WO 95/22058; and U.S. Pat. No. 5,631,734.

In yet another embodiment, the invention contemplates using a panel of antibodies which are generated against the marker polypeptides of this invention, such as polypeptides encoded by one or more of the pCD Gene Sequences. Such a panel of antibodies may be used as a reliable diagnostic probe for IBD. The assay of the present invention comprises contacting a biopsy sample containing cells, e.g., colon cells, with a panel of antibodies to one or more of the encoded products to determine the presence or absence of the marker polypeptides.

The diagnostic methods of the subject invention may also be employed as follow-up to treatment, e.g., quantitation of the level of marker polypeptides may be indicative of the effectiveness of current or previously employed IBD therapies as well as the effect of these therapies upon patient prognosis.

Accordingly, the present invention makes available diagnostic assays and reagents for detecting gain and/or loss of marker polypeptides from a cell in order to aid in the diagnosis and phenotyping of proliferative disorders arising from, for example, tumorigenic transformation of cells.

The diagnostic assays described above can be adapted to be used as prognostic assays, as well. Such an application takes advantage of the sensitivity of the assays of the invention to events which take place at characteristic stages in the progression of the disorder.

The methods of the invention can also be used to follow the clinical course of an IBD. For example, the assay of the invention can be applied to a tissue sample from a patient; following treatment of the patient for the IBD, another tissue sample is taken and the test repeated. Successful treatment will result in either removal of all cells which demonstrate differential expression characteristic of the IBD.

In yet another embodiment, the invention provides methods for determining whether a subject is at risk for developing a disease, such as a predisposition to develop IBD, for example UC or CD, associated with an aberrant activity of any one of the polypeptides encoded by nucleic acids of an IBD gene, such as a pCD Gene Sequence, wherein the aberrant activity of the polypeptide is characterized by detecting the presence or absence of a genetic lesion characterized by at least one of (i) an alteration affecting the integrity of a gene encoding a marker polypeptides, or (ii) the mis-expression of the encoding nucleic acid. To illustrate, such genetic lesions can be detected by ascertaining the existence of at least one of (i) a deletion of one or more nucleotides from the nucleic acid sequence, (ii) an addition of one or more nucleotides to the nucleic acid sequence, (iii) a substitution of one or more nucleotides of the nucleic acid sequence, (iv) a gross chromosomal rearrangement of the nucleic acid sequence, (v) a gross alteration in the level of a messenger RNA transcript of the nucleic acid sequence, (vii) aberrant modification of the nucleic acid sequence, such as of the methylation pattern of the genomic DNA, (vii) the presence of a non-wild type splicing pattern of a messenger RNA transcript of the gene, (viii) a non-wild type level of the marker polypeptide, (ix) allelic loss of the gene, and/or (x) inappropriate post-translational modification of the marker polypeptide.

The present invention provides assay techniques for detecting lesions in the encoding nucleic acid sequence. These methods include, but are not limited to, methods involving sequence analysis, Southern blot hybridization, restriction enzyme site mapping, and methods involving detection of absence of nucleotide pairing between the nucleic acid to be analyzed and a probe.

Specific diseases or disorders, e.g., genetic diseases or disorders, are associated with specific allelic variants of polymorphic regions of certain genes, which do not necessarily encode a mutated protein. Thus, the presence of a specific allelic variant of a polymorphic region of a gene in a subject can render the subject susceptible to developing a specific disease or disorder. Polymorphic regions in genes, can be identified, by determining the nucleotide sequence of genes in populations of individuals. If a polymorphic region is identified, then the link with a specific disease can be determined by studying specific populations of individuals, e.g, trig individuals which developed a specific disease, such as an IBD. A polymorphic region can be located in any region of a gene, e.g., exons, in coding or non coding regions of exons, introns, and promoter region.

In an exemplary embodiment, there is provided a nucleic acid composition comprising a nucleic acid probe including a region of nucleotide sequence which is capable of hybridizing to a sense or antisense sequence of a gene or naturally occurring mutants thereof, or 5′ or 3′ flanking sequences or intronic sequences naturally associated with the subject genes or naturally occurring mutants thereof. The nucleic acid of a cell is rendered accessible for hybridization, the probe is contacted with the nucleic acid of the sample, and the hybridization of the probe to the sample nucleic acid is detected. Such techniques can be used to detect lesions or allelic variants at either the genomic or mRNA level, including deletions, substitutions, etc., as well as to determine mRNA transcript levels.

A preferred detection method is allele specific hybridization using probes overlapping the mutation or polymorphic site and having about 5, 10, 20, 25, or 30 nucleotides around the mutation or polymorphic region. In a preferred embodiment of the invention, several probes capable of hybridizing specifically to allelic variants are attached to a solid phase support, e.g., a “chip”. Mutation detection analysis using these chips comprising oligonucleotides, also termed “DNA probe arrays” is described e.g., in Cronin et al. Human Mutation 7:244 (1996). In one embodiment, a chip comprises all the allelic variants of at least one polymorphic region of a gene. The solid phase support is then contacted with a test nucleic acid and hybridization to the specific probes is detected. Accordingly, the identity of numerous allelic variants of one or more genes can be identified in a simple hybridization experiment.

In certain embodiments, detection of the lesion comprises utilizing the probe/primer in a polymerase chain reaction (PCR) (see, e.g. U.S. Pat. Nos. 4,683,195 and 4,683,202), such as anchor PCR, Reverse transcription PCR (RT-PCR) or RACE PCR, or, alternatively, in a ligase chain reaction (LCR) (see, e.g., Landegran et al. Science 241:1077-1080 (1988); and Nakazawa et al. Proc. Natl. Acad. Sci. USA 91:360-364 (1994)), the latter of which can be particularly useful for detecting point mutations in the gene (see Abravaya et al. Nuc. Acid. Res. 23:675-682 (1995)). In a merely illustrative embodiment, the method includes the steps of (i) collecting a sample of cells from a patient, (ii) isolating nucleic acid (e.g., genomic, mRNA or both) from the cells of the sample, (iii) contacting the nucleic acid sample with one or more primers which specifically hybridize to a nucleic acid sequence under conditions such that hybridization and amplification of the nucleic acid (if present) occurs, and (iv) detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Alternative amplification methods include: self sustained sequence replication (Guatelli et al., Proc. Natl. Acad. Sci. USA 87:1874-1878 1990), transcriptional amplification

system (Kwoh et al., Proc. Natl. Acad. Sci. USA 86:1173-1177 (1989)), Q-Beta Replicase (Lizardi et al., Bio/Technology 6:1197 (1988)), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In a preferred embodiment of the subject assay, mutations in, or allelic variants, of a gene from a sample cell are identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Pat. No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

Another aspect of the present invention relates to methods for identifying genes which are up- or down-regulated in intestinal tissue of patients who have, or are at risk of developing, an inflammatory bowel disease or disorder. In general, the method provides for

(i) generating a first library of nucleic acid probes representative of genes expressed by intestinal tissue of an animal without apparent symptoms and/or risk for an inflammatory bowel disease or disorder;

(ii) generating a second library of nucleic acid probes representative of genes expressed by intestinal tissue of an animal which has symptoms of, and/or is at risk for developing, an inflammatory bowel disease or disorder; and

(iii) identifying genes that up- or down-regulated, e.g., by at least a predetermined fold difference, in the second library of nucleic acids relative to the first library of nucleic acids.

The subject method can include such further steps as: cloning those genes which are up- or down-regulated; generating nucleic acid probes for detecting the level of expression of those genes which are up- or down-regulated; and providing kits, such as microarrays, including probes for detecting the level of expression of those genes which are up- or down-regulated.

In one preferred embodiment, the present invention relates to methods of determining the phenotype of a cell, particularly a cell of intestinal origin, comprising detecting the differential expression, relative to a normal cell, of at least one gene (and more preferably 10, 25 or even 50 different genes) of the pCD Gene Sequences or other IBD genes identified according to the subject differential display methodology. In particular, the present invention provides methods of determining the phenotype of a cell, particularly a cell of intestinal origin, comprising detecting the differential expression, relative to a normal cell, or at least one gene, or at least about two genes, about four genes, about six genes, about

eight genes, about ten genes, about twelve genes, about fourteen genes, about sixteen genes, about eighteen genes, or about twenty genes; and more preferably about twenty-five genes, about thirty genes, about thirty-five genes, about forty genes, about forty-five genes, or about fifty genes. The assay detects a difference in the level of expression of at least a factor of two, preferably by at least a factor of five, and more preferably by at least a factor of twenty, or at least a factor of fifty. In particular, wherein the assay detects a difference in the level of expression of at least a factor of about two, about four, about six, about eight, about ten, about twelve, about fourteen, about sixteen, about eighteen, or about twenty; and more preferably a factor of about twenty-five, about thirty, about thirty-five, about forty, about forty-five, or about fifty. In certain embodiments, a change in the level of expression of at least 10 percent, and more preferably at least 25, 50, 75, or 90 percent, of the IBD gene set indicates an increased risk of the patient having, or developing, an inflammatory bowel disease. In preferred embodiments, the changes (up- or down-regulation) of IBD genes which indicate an increased risk of the patient having, or developing, an inflammatory bowel disease are in the same direction, and more preferably of the same approximate magnitude.

In other embodiments, the assay can be used to detect mutations or epigenetic changes effecting the chromosomal integrity of an IBD gene, e.g., by detecting mutations (insertions, deletions, point mutations, methylation levels) to the coding sequence or transcriptional regulatory sequences and, e.g., effecting one or more alleles of an IBD gene. In still other embodiments, the method can be used to detect alterations in splicing of IBD transcripts, changes in the levels of IBD proteins, changes in post-translational modification of IBD proteins, and/or changes in half-lives for IBD proteins.

In addition to detecting alterations at the nucleic acid level, the subject method can be carried out by detecting the level of protein encoded by an IBD gene, e.g., by immunoassay or other proteometric technique.

The subject method can be used diagnostically, e.g., to identify patients who have developed, or are at risk of developing, an inflammatory bowel disease. In this regard, the subject method can also be used to distinguish the cause of inflammatory bowel symptoms, e.g., to distinguish between UC and CD. The subject method can also be used prognostically for patients already diagnosed with an IBD, e.g., to determine the aggressive or stage of their disease. In either case, the subject method can be used to augment treatment decisions.

The samples used to determine the level of expression of an IBD gene or gene product can include biopsied materials. However, in certain embodiments, genes which are up- or down-regulated in inflammatory bowel diseases encode proteins which can be detected in bodily fluids or in fecal matter. For example, as described in further detail below, certain of the IBD genes encode secreted factors. Accordingly, the present invention

specifically contemplates assays which detect a change in the serum level (or other bodily fluid) of one or more secreted IBD gene products. In such embodiments, the method may make use of an immunoassay, e.g., including an antibody panel (or other binding protein) to detect the level of an IBD gene product in the fluid sample.

Another aspect of the present invention provides libraries of nucleic acid probes ("IBD probes") for indexing the level of expression of one or more IBD genes. For instance, such nucleic acid probes can be immobilized on a solid support, e.g., paper, membranes, filters, chips, pins or glass slides, or any other appropriate substrate. In preferred embodiments, the invention provides a microarray of IBD probes for detecting transcripts of at least 5 different IBD genes, more preferably at least 10, and even more preferably at least 25, 50, 75, 100, 125 or all of the genes in the IBD gene set described herein. In particular, the present invention provides a microarray of IBD probes for detecting transcripts of at least about five different IBD genes, about seven different IBD genes, about nine different IBD genes, about thirteen different IBD genes, or about fifteen different IBD genes; preferably at least about twenty different IBD genes, about twenty-five different IBD genes, about thirty different IBD genes, about thirty-five different IBD genes, about forty different IBD genes, about forty-five different IBD genes, or about fifty different IBD genes; and more preferably at least about sixty different IBD genes, about seventy different IBD genes, about eighty different IBD genes, about ninety different IBD genes, about one hundred different IBD genes, or all of the genes of the IBD gene set.

In general, the subject IBD probes will be isolated nucleic acids (oligonucleotides) comprising a nucleotide sequence which hybridizes under stringent conditions to a pCD Gene Sequence or a sequence complementary thereto. In a related embodiment, the nucleic acid is at least about 80% or about 100% identical to a sequence corresponding to at least about 12, at least about 15, at least about 25, or at least about 40 consecutive nucleotides up to the full length of one of the pCD Gene Sequences or a sequence complementary thereto or up to the full length of the gene of which said sequence is a fragment. In certain embodiments, a nucleic acid of the present invention includes at least about five, at least about ten, or at least about twenty nucleic acids from a novel coding sequence region of an IBD gene. The IBD probes may include a label group attached thereto and able to be detected. The label group may be selected from radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors.

In certain embodiments, the kit may further include instructions for using the kit, solutions for suspending or fixing the cells, detectable tags or labels, solutions for rendering a nucleic acid susceptible to hybridization, solutions for lysing cells, or solutions for the purification of nucleic acids.

j. Drug Screening

Still another aspect of the present invention provides drug screening assays for identifying agents which can be used to treat or manage the effects of an inflammatory bowel disease or disorder, e.g., by counteracting the effects of the up- or down-regulation of one or more of the subject IBD genes, such as the pCD Gene Sequences. Such assays include formats which detect agents that inhibit or potentiate expression (transcription or translation) of an IBD gene, formats which detect agents that inhibit or potentiate an activity of an IBD gene product (enzymatic activity, protein-protein interaction, protein-DNA interaction, etc), formats which detect agents that which alter the splicing of IBD gene transcripts, and formats which detect agents that which shorten or extend the half-life of an IBD gene product. For each of the assay embodiments set out above, the assay is preferably repeated for a variegated library of at least 100 different test compounds, though preferably libraries of at least 10^3, 10^5, 10^7, and 10^9 compunds are tested. The test compound (or test agents) can be, for example, peptides, carbohydrates, nucleic acids and other small organic molecules, and/or natural product extracts, such as, but not limited to small molecules, peptide, peptidomimetic, natural compound, siRNA, anti-sense nucleic acid, aptamer, or antibodies and other specific affinity binders.

In yet another aspect, the invention provides pharmaceutical compositions including agents, e.g., which have been identied by the assays described herein, which alter the level of expression or splicing of one or more IBD genes, alter the activity or half-life of an IBD gene product, or which alter the post-translational modification of an IBD gene product.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning — A Laboratory — Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press:1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Pat. No. 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology,

Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Another aspect of the invention is directed to the identification of agents capable of modulating the growth state of an IBD stem cell or its progeny, where the drug screening method involves contact the cell or resulting in vitro tissue (such as 3-D muscosa) with one or more candidate agents, including small organic molecules, nucleic acids, peptides and polypeptides, natural extracts and carbohydrates (as examples). In this regard, the invention provides assays for detecting changes at the cellular level, such as by gross physiological consequences (proliferation, differentiation, cell death) or specified changes, such as modulation of the expression of marker nucleic acids (such as a pCD Gene Sequence) or protein it encodes, changes in expression of a reporter construct, or other detectable discrete changes in the cells morphology, gene expression profil and/or epigenetic profile. The availability according to the present invention of normal tissues and cells, particularly patient matched normal tissue (stem cells and differentiated cells), for counter-screening makes this approach particularly robust.

Exemplary high through out screening using whole cell assays of the IBD stem cells or differentiated tissue include synthetic lethality screens, such as screens using shRNA/siRNA/CRISPR libraries for loss-of-function assays to identify genes for which inhibition is synthetically lethal to IBD stem cell. The nucleic acid library can, in certain embodimetns, be biased to identify cell surface proteins and channels (GPCRs, etc) for which antagonist antibodies and small molecule ligands can be generated, or biased to identify enzyme classes for subsequent small molecule inhibitor development.

Another embodiment of the whole cell drug discovery platform involves differential expression profiling to identify genes/gene products up or down regulated in the IBD stem cell or differentiated tissue thereof which may be potential targets for drugs that can reverse or diminish the role of IBD stem cells in inflammatory bowel diseases. Targets upregulated in IBD stem cells or their progeny, particular those have extracellular cell surface domains, are also particularly amenable to targeting in the development of Antibody-Drug Conjugate agents. Intracellular targets, particularly signal transduction pathways and transcriptional factors, that may be upregulated in the IBD stem cells or differentiated tissue derived therefrom are also potential targets for drugs that can reverse or diminish the role of IBD stem cells in inflammatory bowel diseases, especially small molecule and nucleic acid drug agents. Enzymes, such as proteases, kinases, phosphatases and the like, that may be upregulated in the IBD stem cells or differentiated tissue derived therefrom can be targets for drugs that can reverse or diminish the role of IBD stem cells in inflammatory bowel diseases, especially small molecules and antibodies. Cell surface receptors and channels, such as ligand binding receptors and ion channels, that may be upregulated in the IBD stem cells or

differentiated tissue derived therefrom can be targets for drugs that can reverse or diminish the role of IBD stem cells in inflammatory bowel diseases, especially small molecules and antibodies and ligand antagonists.

Several in vitro cell based methods can be used to identify compounds that modulate expression of the marker nucleic acids (e.g., an IBD gene) and/or alter for example, inhibit the bioactivity of the encoded polypeptide. Merely to illustrate, in certain embodiments drug screening is performed by adding a test compound to a sample of IDB stem cells, and than monitoring the effect. A parallel sample which does not receive the test compound is also monitored as a control. To find agents that are selective for IBD stem cells, a third set of stem cells – derived from nomal GI epithelia – are also monitored with and without adding the test compound. The treated and untreated cells are then compared by any suitable phenotypic criteria, including but not limited to microscopic analysis, viability testing, ability to replicate, histological examination, the level of a particular RNA or polypeptide associated with the cells, the level of enzymatic activity expressed by the cells or cell lysates, and the ability of the cells to interact with other cells or compounds, cell viability, epigenetic changes, etc.. Differences between treated and untreated cells indicates effects attributable to the test compound. A greater difference in responsiveness in the case of the IBD stem cells relative to the normal GI epithelial stem cells indicates selectivity.

Desirable effects of a test compound include an effect on any phenotype that was conferred by the IBD-associated marker nucleic acid sequence. Examples include a test compound that limits the overabundance of mRNA, limits production of the encoded protein, or limits the functional effect of the protein. The effect of the test compound would be apparent when comparing results between treated and untreated cells.

High throughput screening (HTS) is used for analyzing many discrete compounds in parallel, so that large numbers of test compounds can be quickly screened. The most widely established techniques utilize 96- well, 384-well or 1536- well microtiter plates. The wells of the microtiter plates typically require assay volumes that range from 5 to 500 microliters. In addition to the plates, many instruments, materials, pipettors, robotics, plate washers, and plate readers are commercially available to fit the micro well formats.

The chemical entity or biological product is preferably, but not necessarily a low molecular weight compound, but may also be a larger compound, or any organic or inorganic molecule effective in the given situation, including modified and unmodified nucleic acids such as antisense nucleic acids, RNAi, such as siRNA or shRNA, CRISPR-Cas systems, peptides, peptidomimetics, receptors, ligands, and antibodies, aptamers, polypeptides, nucleic acid analogues or variants thereof. Examples include an oligomer of nucleic acids, amino acids, or carbohydrates including without limitation proteins, oligonucleotides, ribozymes, DNAzymes, glycoproteins, siRNAs, lipoproteins, aptamers, and

modifications and combinations thereof. Agents can be selected from a group comprising: chemicals; small molecules; nucleic acid sequences; nucleic acid analogues; proteins; peptides; aptamers; antibodies; or fragments thereof. A nucleic acid sequence can be RNA or DNA, and can be single or double stranded, and can be selected from a group comprising; nucleic acid encoding a protein of interest, oligonucleotides, nucleic acid analogues, for example peptide - nucleic acid (PNA), pseudo-complementary PNA (pc-PNA), locked nucleic acid (LNA), modified RNA (mod-RNA), single guide RNA etc. Such nucleic acid sequences include, for example, but are not limited to, nucleic acid sequence encoding proteins, for example that act as transcriptional repressors, antisense molecules, ribozymes, small inhibitory nucleic acid sequences, for example but are not limited to RNAi, shRNAi, siRNA, micro RNAi (mRNAi), antisense oligonucleotides, CRISPR guide RNA, for example that target a CRISPR enzyme to a specific DNA target sequence etc. A protein and/or peptide or fragment thereof can be any protein of interest, for example, but are not limited to: mutated proteins; therapeutic proteins and truncated proteins, wherein the protein is normally absent or expressed at lower levels in the cell. Proteins can also be selected from a group comprising; mutated proteins, genetically engineered proteins, peptides, synthetic peptides, recombinant proteins, chimeric proteins, antibodies and other specific affinity binders (including humanized antibodies, chimeric antibodies and the like), modified proteins and fragments thereof. Alternatively, the agent can be intracellular within the cell as a result of introduction of a nucleic acid sequence into the cell and its transcription resulting in the production of the nucleic acid and/or protein modulator of a gene within the cell. In some embodiments, the agent is any chemical, entity or moiety, including without limitation synthetic and naturally-occurring non-proteinaceous entities. In certain embodiments the agent is a small molecule having a chemical moiety. Agents can be known to have a desired activity and/or property, or can be selected from a library of diverse compounds.

Cell-based assays for HTS can include such types as second messenger assays, reporter gene assays, and cell proliferation or cell differentiation assays.

2nd messenger	Detect ability of test agent to alter (increase or decrease) signal transduction pathways following activation of cell-surface receptors or other signaling pathways
	Examples: Using fluorescent molecules that respond to changes in intracellular Ca2+ concentration, membrane potential, pH, etc. to assay

	receptor stimulation and ion channel activation
Reporter gene	Detect ability of test agent to alter (increase or decrease) cellular responses at the transcription/translation level of one or more target genes.
	Examples: Coexpression of luciferase to catalyze the light-emitting luciferin reaction for detection of protein kinase C inhibitors Quantification of G-protein coupled receptor (GPCR) internalization using a GPCR-green fluorescent protein hybrid
Cell proliferation/differentiation cytotoxicity	Detect ability of test agent to alter (increase or decrease) the overall cell growth or death in response of IBD stem cells to external stimuli or stress. Detect ability of test agent to alter (increase or decrease) differentiation of IBD stem cells, e.g., under air-liquid interface conditions, to produce diseased or normal epithelial cells/tissues
Epigenetic State	Detect ability of test agent to alter (increase or decrease) epigenetic state of IBD stem cell or differentiated epithelial cells/tissue derived from the IBD stem cell, such as for changes in chromatin structure (such as by chromatin immunoprecipitation), and/or DNA methylation (such as by bisulfite modification or CpG island microarray).

The majority of cell-based HTS assays are carried out in multi-well plates as they can be easily miniaturized to increase the number of wells per plate for high throughput rates, on the order of 10,000 compounds per assay per day, and handled with a robotic system for automation. More recently, there are increasing interests in developing microfluidic devices

for perfusion cultures that allow for the evaluation of long-term drug effects as well as studying interactions among different cell types in a biological system like the whole animal.

In general, cell culture modes include single cells, monolayer cells on a two-dimensional surface, multilayer cells or aggregate clusters in a 3D scaffold. 2D cell-based assays in multiwell plates together with automated operation are widely used in drug screening because of their low costs and easy operation. The third dimension in a 3D scaffold provides another direction for cell-cell interactions, cell migration, and cell morphogenesis, which may be critical in regulating cell cycle and tissue functions of the IBD stem cells or their differentiated progeny. In addition, 3D cell cultures provide not only the templates for cells to adhere and grow, but also the interconnectivity within the 3D constructs to allow nutrients and metabolites to be transported in and out of the engineered tissues. Consequently, 3D cell cultures may support a higher cell density than 2D cell cultures. In certain embodiments, such as differentiated tissues derived from the IBD stem cells, high specific surface areas offered by 3D also allow for a long-term cell culture in vitro.

Widely used HTS platforms (e.g. 96-, 384-, 1536-well plates) offer static microenvironments, with the medium supplied in a batch-wise manner. Although automation using robots allows the static cultures to be used as a feasible HTS platform for drug screening, in certain instances static cultures is not ideal for long-term cell culture due to the risk of contamination caused by repeated interventions. Modified multiwell plates with the integration of microfluidic systems, which has been reported with high throughput for drug screening (Kim et al. 2004 Breast Cancer Res Treat, 85:281-291) and cytotoxicity evaluation of anticancer drugs (Cukierman et al. 2001 Science 294:1708-1712) can therefore also be readily adapted for use in the drug screening embodiments of the present invention. Such systems, where a perfusion cell culture is achieved to compensate liquid evaporation, can maintain a cell culture for an extended period for testing long-term effects of drugs.

In addition to continuously providing nutrients and waste removal and thus keeping the cell culture system stable, perfusion can also be used to generate gradients of drug concentrations, creating a specific physical microenvironment (e.g. shear stress or interstitial fluid flow) and constructing a circulatory system to better mimic the in vivo conditions. See Heldin et al. 2004 Nat Rev Cancer 4:806-813. Compared to the static cell culture, perfusion can increase cell content and matrix synthesis in a 3D IBD tissue system derived by differentiation of IBD stem cells.

Cell-based assays are well established and widely used to analyze the effects of compounds on cellular activities, including nuclear size, mitochondrial membrane potential, intracellular calcium levels, membrane permeability, and cell number. The failure of early identification of toxic side effects of a compound has resulted in about 30% of the attrition of new drug candidates. Therefore, cytotoxicity testing, which generally relies on the

quantification of cell number and viability, has become one of the most critical steps in early-phase drug discovery. Conventional methods for cell number counting use hemacytometer, Coulter counter or flow cytometry can be labor-intensive and time consuming, while Trypan blue exclusion and neutral red uptake methods for determining cell viability require the use of invasive chemicals. Furthermore, these methods have a relatively low throughput, and thus are not always good choices for HTS though can be used in the drug screening assays of the present invention. As off-line sampling during the cell culture process is limited by the small amount of medium used in HTS assays, from several µl to several ml, online detection is desired. Detection methods used in cell-based HTS assays can be divided mainly into two groups: electrochemical and optical techniques. In general, optical sensing is easier for miniaturization than electrochemical sensing.

(i) Electrochemical Methods

Various electrochemical biosensors, which integrate biological recognition elements and electrochemical transduction units, based on (a) cellular activity and function; (b) cellular barrier behavior; and (c) recording/stimulation of electric potential of electrogenic cells have been developed. These systems can generally adapted for use with the subject IBD stem cells and/or differentiated tissues derived therefrom, and be used to achieve noninvasive online monitoring of drug effects – such as cell killing/toxicity or alterations to proliferation rates or phenotypes/genotypes resulting from differentiation or epigenetic changes.

(ii) Electrochemical Method Based on Cellular Activity and Function

A living cell can be considered as an electrochemical system. Electron generation and charge transfer caused by redox reactions and the changes of ionic composition and concentration in living cells can be used to characterize cell viability in a homogenous solution. For example, when the IBD stem cells or their progeny are attached to a gold nanoparticle-modified carbon paste electrode, with platinum wire as auxiliary and saturated calomel electrode as reference electrodes, the cells can exhibit an irreversible voltammetric response which is related to the oxidation of guanine. The oxidation peak can be used to investigate the exogenous effect of a test agent on the oxidative state or resistant to oxidative damage to the IBD stem cell or IBD tissue, which provides an electrochemical approach for studying IBD stem cell drug sensitivity.

In addition, metabolism in cells leads to changes in metabolic products (e.g., lactic acid and carbon dioxide) or substrates (e.g., glucose and dissolved oxygen [DO]). A variety of electrochemical biosensors based on metabolic changes have been fabricated and can be used to test the ability of a test agent (or agents) to alter the metabolic state of an IBD stem

cell or tissue derived therefrom. Electrochemical methods based on cellular activities include potentiometry and amperometry.

Conventional potentiometry cell-based sensors include an ion-selective electrode (ISE) or gas-sensing electrode (GSE) coated with a layer of cells. An ISE has been developed for screening of toxins by integrating cells with a K+ selective film. In such systems, a potential change caused by the ion accumulation or depletion on the electrode surface can be used to monitor metabolic products during cell growth.

Amperometric electrochemical methods using a specific enzyme electrode are widely used for the determination of pH, DO or glucose. The acidification rate in the vicinity of cells can be quantified using a microphysiometer. Cellular biochemical responses resulting from the accumulation of lactic acid and carbon dioxide can be approximately monitored using the pH value in pH-sensing chambers. Furthermore, heterogeneous pO2 distributions around tissues could be detected using a miniaturized system.

(iii) Electrochemical Method Based on Barrier Behavior

The local ionic environment at the electrode/solution interface changes in the presence of cells. In general, cells with insulating properties would significantly increase the electrode impedance. Thus, biological status of cells, including cellular viability, morphology, cell number, and cell apoptosis, and cell adhesion can be monitored using electrochemical impedance spectroscopic techniques. For example, an electrical impedance sensor array integrated into the bottom of a microtiter plate has been developed for the quantitative detection of living cells. Real-time assessment of cytotoxicity and acute toxicity can be achieved using this device. See Ku et al. 2008 Anal Chem 80:7543-7548.

(iv) Electrochemical Method Based on Cellular Electrical Potential

Electrogenic cells and tissues, such as heart muscle, pancreas beta and nerve cells, are able to generate bioelectrical signals resulting from the orchestrated activities of ion channels embedded within cell membrane. The normal epithelial lining of the gut, such as of the terminal ileum, undergo electrogenic sodium absorption that can create a signal for detection. These bioelectrical signals can be used to test drugs against critical diseases such as electrogenic secretion (Chlorine) and/or absorption (Sodium, Potassium) in tissue differentiated from normal versus IBD stem cells. For example, detection can make use of a nanoelectronic biosensor based on single-wall carbon nanotubes (SWCNTs). This method can be used to non-invasively detect cellular activities for electrogenic cells derived from the IBD stem cells with high throughput, high sensitivity, easy use, and the capacity of long-term cell culture.

(v) Optical Methods

Optical detection in cell-based HTS assays usually is carried out with colorimetric, luminescent, or fluorescent methods, examples of which are discussed below.

(vi) Colorimetric Method

Colorimetric methods are based on color change of the growth medium after cell metabolites react with chemical agents. Colorimetric assays using ruthenium dye and Alamar Blue, for example, have been developed and are readily adapted for use with the present invention. In addition, a spectrum of assays using tetrazolium salts such as MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) are also commercially available. These methods are based on the reduction of a tetrazolium salt by actively growing cells to a colored formazan product that can be quantified with a spectrophotometer.

(vii) Luminescent Methods

Many organisms, including fireflies and some marine organisms, regulate their light production using luciferase in a variety of light-emitting reactions with emission color ranging from yellow-green to red. In luminescent assays, the oxidation of luciferin catalyzed by luciferase produces light that can be detected by a light sensitive apparatus such as an illuminometeror optical microscope, allowing observation of biological processes. Some luminescent reactions are mediated by ATP or calcium ions. Luciferase has been widely used as a reporter in cells expressing a luciferase gene under the control of a promoter of interest to assess its transcriptional activity. Many commercial cell-based kinase activity assays use luciferase as the reporter. Luciferase can also be used to detect the level of cellular ATP in cell viability assays. In addition, some enzymes, such as caspase and cytochrome P450, can convert proluminescent molecules to luciferin, and their activities can thus be detected in a coupled or two-step luciferase assay.

Although firefly luciferase is widely used in cell-based assays, its application in HTS is usually limited to endpoint assays because the requirements of cell lysis and addition of luciferase substrates. Unlike firefly luciferase, the luciferase (MetLuc) derived from the marine copepod Metridia longa is naturally secreted, which allows the development of live cell assays and multiple assays on the same cells using a no-lysis protocol.

(viii) Fluorescent Methods

Compared to luminescent methods, fluorescent methods have higher sensitivity and can be easier to be miniaturized for large-scale or high throughput measurements of cell

activities, pathway activation, toxicity, and phenotypic cellular responses of exogenous stimuli. Fluorescent methods for cell-based assays were initially developed using small, highly-fluorescent, organic molecules, monitoring ion concentrations, membrane potential and as intracellular substrates for reporter genes. More recently, nanoparticles such as quantum dots (QDs) have been widely used as labels in cell-based assays and be adapted for use in the present invention. These semiconductor nanocrystals are photo-chemically stable, can provide a narrow and adjustable emission, and can be excited by light of any wavelength shorter than that of the emission peak. Thus, various emission colors can be simultaneously obtained using nanoparticles of different sizes excited with a single-wavelength light.

The development of reporter gene techniques using green fluorescent protein (GFP) has enabled online, non-invasive detection and quantification of cell proliferation and specific cellular functions. GFP, which was first discovered in the jellyfish, and its mutants have been developed with emission light colors ranging from blue to yellow. GFP is species-independent and generally non-toxic to cells. Its detection can be performed in living samples. Therefore, IBD stem cell-based assays using GFPs are amenable to real-time, automated, and non-invasive assessment of both chronic and acute cellular events. In addition, GFP can be coupled with Disco soma species red fluorescent protein (dsRed) for two-color or multiplex assays.

Cell-based assays using cDNA encoding a fluorescent protein provide an HT platform for non-invasive analysis of cell proliferation and death kinetics. Since a specific cellular event or function can be monitored based on the regulatory DNA sequence or promoter used in controlling the expression of the reporter gene, the assay is responsive to targeted effects, such as activation of signal transduction pathways, and is suitable for use in disease-relevant assays. In addition, two fluorescent proteins fused with a peptide linker comprising a caspase-3 cleavage site can be used to study the activation of caspase-3 or apoptosis in live cells based on changes in emission wavelength due to energy transfer between two close fluorophores, a phenomenon called fluorescence resonance energy transfer (FRET).

In general, whole-cell autofluorescence-based systems are non-invasive, fast, and simple for HTS applications. It can provide dynamic data and be used as high-content assays as well. Current commercial HTS systems use laser scanning imaging systems with fluorescence microscopy and quantitative image analysis to perform live-cell kinetic assays with high spatial and temporal resolution, and the various IBD stem cell and differentiated progeny thereof can be adapted for drug screening with these systems. They can be used to examine the context of living cells, quantify intracellular proteins, and monitor the trafficking of proteins fused with fluorescent reporters and some subcellular structures.

(ix) Microfluidic Cell-Based Assays

Microfluidcs has emerged as a promising technology with widespread applications in engineering, biology and medicine. It has the potential to revolutionize the way we approach cell biology research. Microfluidics refers to the science and technology that allows one to manipulate tiny amounts (nanoliter to microliter) of fluids using microstructures with characteristic dimensions on the order of tens to hundreds of micometers. The controllable processing of microfluidic devices at dimensions close to cells and biomolecules enable their biological applications at the cellular level. In addition, the scale of microchannels corresponds well with the native cellular microenvironments, in which the ratio of cell volume to extracellular fluid volume can be greater than one.

Microfabricated cell culture devices have previously been demonstrated on silicon and polydimethylsiloxane (PDMS) substrates with other cells, and can be used with IBD stem cells and cells/tissues differentiated therefrom. Most of microfluidic platforms are fabricated using PDMS, which is optically transparent, gas permeable and biocompatible. The conventional format of microfluidics is not an ideal match for complete cell culture, because all of the reagents and cells are positioned in an interconnected network of enclosed microchannels, making it difficult to establish fresh, sterile sites for seeding new generations of cells. Recently, digital microfluidics (DMF) has emerged as an alternative to the conventional format of enclosed microchannels. DMF is a technique, in which nanoliter-sized droplets are manipulated on an open surface of an array of electrodes. For example, the lab-on-a-chip platforms are available which are capable of implementing all of the steps required for mammalian cell culture: cell seeding, growth, detachment, and re-seeding on a fresh surface for complete mammalian cell culture.

(x) Animal Models

The subject IBD stem cells or tissue derived therefrom can be used to generate non-human animals (preferably mammals) for in vivo evaluation of test agents. As shown in Figure 22, IBD stem cells can be directed injected in sites within or outside of the gut, and the ability of a test agent to alter the growth, survival, differentiation of the IBD stem cells or tissue, and/or the recruitment of fibroblasts, immune cells or other cells to the site of the injection of the IBD stem cells or tissue can be evaluated.

In certain embodiments, the animal is a non-human primate. In other embodiments, the animal is a rodent, such as a mouse or rat. The animal may also be a rabbit, dog, cat, pig, cow, or other suitable non-human mammal for testing.

In certain embodiments, the IBD stem cells or derived tissue are of human origin, and the animal is immunosuppressed or immuno-incompetent, such as a SCID, NUDE or NIHIII mouse.

In certain embodiments, the IBD stem cells or derived tissue are introduced into the animal with a naturally occurring or artificial extracellular matrix (ECM). Examples of extracellular matrix -producing cells include chondrocytes that mainly produce collagen and proteoglycans; fibroblast cells that mainly produce type IV collagen, laminin, interstitial procollagens, and fibronectin; and colonic myofibroblasts that mainly produce collagens (type I, III, and V), chondroitin sulfate proteoglycan, hyaluronic acid, fibronectin, and tenascin-C. Alternatively, the ECM is a MATRIGEL basement membrane matrix.

(xi) Formulation and Use of Discovered Drug Agents

Subsequent to indetifying an agent that selectively effects the IBD stem cell or tissue derived from the IBD stem cell, relative to normal gut epithelial stem cells or tissue, the agent can be further optimized (such as by structure-activity alterations to the chemical structure, amino acid changes, truncations, fusions and other modifications in the case of protein or peptide therapeutics), subjected to animal toxicity and efficacy testing, utilized in human clinical trials, and ultimately, if approved for marketing, the resulting drug agent formulated for use in human patients and commercialized for use (administration) to human patients. That is, the present invention provides a method for providing a drug for treating inflammatory bowel disease, such as Crohn's diease or ulcerative colitis, comprising an overall discovery process for the drug which includes: contacting one or more test agents (e.g., in vitro) with IBD stem cells of the present invention or tissue derived therefrom, identifying a test agent, herein a "drug agent", that alters, for example, the proliferation, differentiation, epigenetic and/or genotypic state of the stem cell or tissue (preferably selectively relative to normal intestinal stem cells or tissue); optionally generating modifications to the structure of the drug agent to create an improved drug agent; establishing the safety and (optinally) efficacy of the drug agent (or improved drug agent) in non-human animals; formulating the drug agent or improved drug agent for use in human patients; conducting clinical trials in human subjects establishing the safety and efficacy of the drug agent or improved drug agent in human subjects; generating a packaged pharmaceutical preparation comprising the drug agent or improved drug agent formulated for use in human patients and a label providing instructions for administering and discontinuing use of the drug agent or improved drug agent in human patients.

In certain embodiments, the drug agent is selective for IBD stem cells or tissue derived from the IBD stem cell, relative to normal gut epithelial stem cells or tissue, by a

factor of at least 2, more preferably at least 5, 10, 20, 30, 40, 50, 75, 100, 250, 500 or at least a 1000.

In certain embodiments, the drug agent is selective for IBD stem cells or tissue derived from the IBD stem cell, relative to normal gut epithelial stem cells or tissue, having an EC50 for the biological effect on IBD stem cells or tissue derived therefrom at least 2-fold less relative to EC50 for biological effect on normal gut epithelial stem cells or tissue, more preferably at least 5, 10, 20, 30, 40, 50, 75, 100, 250, 500 or at least a 1000 less.

In certain embodiments, the drug agent is selective for IBD stem cells or tissue derived from the IBD stem cell, relative to normal gut epithelial stem cells or tissue, having an IC50 for killing IBD stem cells or tissue derived therefrom at least 2-fold less relative to IC50 for killing on normal gut epithelial stem cells or tissue, more preferably at least 5, 10, 20, 30, 40, 50, 75, 100, 250, 500 or at least a 1000 less.

Pharmaceutical compositions of the present invention can be formulated to be administered orally, rectally, parentally, including, intravenously, mucosally, subcutaneously, intranasally, via inhalation (e.g., aerosol inhalation), locally, infusion, via a catheter, via a lavage, or by any other method or any combination of the foregoing as would be practiced by one of ordinary skill in the art.

In another embodiment, the invention provides a method for conducting a pharmaceutical business, comprising: (a) manufacturing the packaged pharmaceutical of the invention; and (b) marketing to healthcare providers the benefits of using the package or preparation to treat patients suffering from an inflammatory bowel disease such as Crohn's disease or ulcerative colitis.

In another embodiment, the invention provides a method for conducting a pharmaceutical business, comprising: (a) providing a distribution network for selling the packaged pharmaceutical of the invention; and (b) providing instruction material to patients or physicians for using the package or preparation to treat patients suffering from an inflammatory bowel disease such as Crohn's disease or ulcerative colitis.

In another embodiment, the invention provides a method for conducting a pharmaceutical business, comprising: (a) determining an appropriate dosage of an drug agent or improved drug agent identified by the methods of the present invention to treat patients suffering from an inflammatory bowel disease such as Crohn's disease or ulcerative colitis; (b) conducting therapeutic profiling of one or more formulations of the drug agent or improved drug agent identified in step (a), for efficacy and toxicity in animals; and (c) providing a distribution network for selling a the formulations identified in step (b) as having an acceptable therapeutic profile. The method may include an additional step of providing a sales group for marketing the preparation to healthcare providers.

In another embodiment, the invention provides a method for conducting a medical assistance reimbursement program, comprising: (a) providing a reimbursement program which permits, for prescription of a drug agent or improved drug agent of the invention for treating an inflammatory bowel disease, at least partial reimbursement to a healthcare provider or patient, or payment to a drug distributor; (b) processing one or more claims for prescription of a drug agent or improved drug agent of the invention for treating an inflammatory bowel disease; and (c) reimbursing the healthcare provider or patient, or paying a drug distributor, at least a portion of the cost of said prescription.

In another embodiment, the invention provides a method for treating an inflammatory bowel disease comprising administering to the patient a composition of a drug agent or improved drug agent of the invention in an amount sufficient to treat the disease in the animal as evaluated by a standardized test.

k. Transgenic Animals

Another aspect of the present invention relates to transgenic non-human animals having germline and/or somatic cells in which the biological activity of one or more of the IBD Genes Set, such as one or more of the pCD Gene Sequences, are altered by a chromosomally incorporated transgene. Such animals can be used as models for inflammatory bowel diseases or disorders, e.g., for understanding the pathology of disease and/or drug screening.

In one embodiment, the present invention provides a desired non-human animal or an animal (including human) cell which contains a predefined, specific and desired alteration rendering the non-human animal or animal cell predisposed to and inflammatory bowel disease.

In embodiments where the IBD gene is down-regulated in the disease state, the transgene may encode a mutant protein, such as dominant negative protein which antagonizes at least a portion of the biological function of a wild-type protein. Yet in other embodiments, the transgene can encode an antisense transcript which, when transcribed from the transgene, hybridizes with a gene or a mRNA transcript thereof, and inhibits expression of the gene. In still other embodiments, the transgene can, by such mechanisms as homologous recombination, knock-out the endogenous IBD gene.

A preferred transgenic non-human animal of the present invention has germline and/or somatic cells in which one or more alleles of a gene are disrupted by a chromosomally incorporated transgene, wherein the transgene includes a marker sequence providing a detectable signal for identifying the presence of the transgene in cells of the transgenic animal, and replaces at least a portion of the gene or is inserted into the gene or disrupts expression of a wild-type protein.

In embodiments where the IBD gene is up-regulated in the disease state, the transgene may encode a wild-type IBD gene product, and the transcriptionally regualtory sequences of the transgene can be used to cause overexpression of the IBD gene. Likewise, mutant IBD genes can be used which encode IBD proteins that are consitutitively or regulatively activated to mimic overexpression of the endogenous IBD gene.

Still another aspect of the present invention relates to methods for generating non-human animals and stem cells having a functionally disrupted endogenous gene. In a preferred embodiment, the method comprises the steps of:

(i) constructing a transgene construct including (a) a recombination region having at least a portion of an IBD gene, which recombination region directs recombination of the transgene with the gene, and (b) a marker sequence which provides a detectable signal for identifying the presence of the transgene in a cell;

(ii) transfering the transgene into stem cells of a non-human animal;

(iii) selecting stem cells having a correctly targeted homologous recombination between the transgene and the gene;

(iv) transfering cells identified in step (iii) into a non-human blastocyst and implanting the resulting chimeric blastocyst into a non-human female; and

(v) (v) collecting offspring harboring an endogenous gene allele having the correctly targeted recombination.

Yet another aspect of the invention provides a method for evaluating the potential of an agent to cause an IBD or to protect against development of an IBD by (i) contacting a transgenic animal of the present invention with a test agent, and (ii) ascertaining the presence, and more preferably the level, of onset or degree of severity of an inflammatory bowel disease or disorder, and comparing that with an untreated transgenic animal or transgenic animal treated with a control agent.

I. VSIG1 Agents

Given the pronounced overexpression of VSIG1 (V-set and immunoglobulin domain containing 1) in the isolated stem cells of pediatric Crohn's patients, another aspect of the invention relates to the use and pharmaceutical preparations of agents which reduce the expression of the VSIG1 gene or reduce or inhibit the biological activity of the VS1G1 protein, as as well as the detection of VSIG1 gene expression and VSIG1 protein levels as part of diagnostic assays and imaging protocols for identifying the presence of Crohn's stem cells or their progeny in patient samples or in in vivo imaging.

The VSIG1 gene encodes a member of the junctional adhesion molecule (JAM) family. A representative sequence for the human VSIG1 gene is provided in Gene ID: 340547 (see also Ensembl:ENSG00000101842, MIM:300620 and

Vega:OTTHUMG00000022175). The members of immunoglobulin superfamily are transmembrane proteins and most of these proteins are involved in cell-cell adhesion. These proteins are highly glycosylated through N- and O-glycosylation. The encoded protein contains multiple glycosylation sites at the N-terminal region, and multiple phosphorylation sites and glutamic acid/proline (EP) repeats at the C-terminal region. The gene is expressed in normal stomach and testis, as well as in gastric, esophageal and ovarian cancers. Alternatively, spliced transcript variants encoding different isoforms have been found for this gene.

One aspect of the invention provides pharmaceutical preparations that inhibit or reduce the role in disease development or progression of IBD stem cells or their progeny that have upregulated expression of VSIG1, i.e., to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease. These include, with limitation, nucleic acids, peptides and polypeptides (including antibodies), natural products, small molecules and carbohydrates. In certain embodiments, the therapeutic agent is locally delivered to the area of the gut afflicted by IBD, such as by oral delivery or local administered by injection or surgical placement.

In certain embodiments, the invention provides pharmaceutical preparations of nucleic acids that inhibit or reduce the expression of VSIG1 to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease. These include, with limitation, antisense nucleic acids or RNA intereference nucleic acids (such as siRNA, miRNA and shRNA) or a sequence-directed ribozyme which include sequences that hybridize to a portion of the VSIG1 gene sequence or RNA transcript therefrom.

In certain embodiments, the invention provides pharmaceutical preparations of antibodies that selectively bind to and inhibit or reduce the biological function of VSIG1 to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease.

In certain embodiments, the invention provides pharmaceutical preparations of antibody-drug conjugates (ADC) including antibodies that selectively bind to VSIG1 to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease. The targeting of VSIG1 with the antibody portion of the ADC permits the delivery of a therapeutic agent to the diseased portion of the gut. In certain embodiments, the drug component of the ADC is cytotoxic or cytostatic to the cell expressing the VSIG1 protein to which the ADC binds. In other embodiments, the drug component of the ADC inhibits the differentiation and/or epigenetic traits of the the cell expressing the VSIG1 protein to which the ADC binds.

Another aspect of the invention provides diagnostic reagents for detecting the upregulated expression of VSIG1, for example, to identify the presence or absence of IBD

stem cells in a biopsy or to image IBD impacted portions of the gut. These include, with limitation, nucleic acids and VSIG1 binding agents suchas antibodies.

m. CLDN18 Agents

Given the pronounced overexpression of CLDN18 (Claudin-18) in the isolated stem cells of pediatric Crohn's patients, another aspect of the invention relates to the use and pharmaceutical preparations of agents which reduce the expression of the CLDN18 gene or reduce or inhibit the biological activity of the CLDN18 protein, as as well as the detection of CLDN18 gene expression and CLDN18 protein levels as part of diagnostic assays and imaging protocols for identifying the presence of Crohn's stem cells or their progeny in patient samples or in in vivo imaging.

The CLDN18 gene encodes a member of the claudins family, which are integral membrane proteins and components of tight junction strands. Tight junction strands serve as a physical barrier to prevent solutes and water from passing freely through the paracellular space between epithelial or endothelial cell sheets, and also play critical roles in maintaining cell polarity and signal transductions. PKC/MAPK/AP-1 (protein kinase C/mitogen-activated protein kinase/activator protein-1) dependent pathway regulates the expression of this gene in gastric cells. A representative sequence for the human CLDN18 gene is provided in Gene ID: 51208 (see also Ensembl:ENSG00000066405, MIM:609210 and Vega:OTTHUMG00000159762).

One aspect of the invention provides pharmaceutical preparations that inhibit or reduce the role in disease development or progression of IBD stem cells or their progeny that have upregulated expression of CLDN18, i.e., to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease. These include, with limitation, nucleic acids, peptides and polypeptides (including antibodies), natural products, small molecules and carbohydrates. In certain embodiments, the therapeutic agent is locally delivered to the area of the gut afflicted by IBD, such as by oral delivery or local administered by injection or surgical placement.

In certain embodiments, the invention provides pharmaceutical preparations of nucleic acids that inhibit or reduce the expression of CLDN18 to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease. These include, with limitation, antisense nucleic acids or RNA intereference nucleic acids (such as siRNA, miRNA and shRNA) or a sequence-directed ribozyme which include sequences that hybridize to a portion of the CLDN18 gene sequence or RNA transcript therefrom.

In certain embodiments, the invention provides pharmaceutical preparations of antibodies that selectively bind to and inhibit or reduce the biological function of CLDN18 to

be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease. An exemplary antibody for use as a therapeutic agent in the present invention is IMAB362 (Claudiximab), see PCT Publication WO2013174404.

In certain embodiments, the invention provides pharmaceutical preparations of antibody-drug conjugates (ADC) including antibodies that selectively bind to CLDN18 to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease. The targeting of CLDN18 with the antibody portion of the ADC permits the delivery of a therapeutic agent to the diseased portion of the gut. In certain embodiments, the drug component of the ADC is cytotoxic or cytostatic to the cell expressing the CLDN18 protein to which the ADC binds. In other embodiments, the drug component of the ADC inhibits the differentiation and/or epigenetic traits of the the cell expressing the CLDN18 protein to which the ADC binds. An exemplary antibody for use in the antibody-drug conjugates of the present invention is IMAB362 (Claudiximab), see PCT Publication WO2013174404.

Another aspect of the invention provides diagnostic reagents for detecting the upregulated expression of CLDN18, for example, to identify the presence or absence of IBD stem cells in a biopsy or to image IBD impacted portions of the gut. These include, with limitation, nucleic acids and CLDN18 binding agents suchas antibodies.

n. CD74 Agents

Given the pronounced overexpression of CD74 in the isolated stem cells of pediatric Crohn's patients, another aspect of the invention relates to the use and pharmaceutical preparations of agents which reduce the expression of the CD74 gene or reduce or inhibit the biological activity of the CD74 protein, as as well as the detection of CD74 gene expression and CD74 protein levels as part of diagnostic assays and imaging protocols for identifying the presence of Crohn's stem cells or their progeny in patient samples or in in vivo imaging.

The CD74 gene encodes a protein that associates with class II major histocompatibility complex (MHC) and is an important chaperone that regulates antigen presentation for immune response. It also serves as cell surface receptor for the cytokine macrophage migration inhibitory factor (MIF) which, when bound to the encoded protein, initiates survival pathways and cell proliferation. This protein also interacts with amyloid precursor protein (APP) and suppresses the production of amyloid beta (Abeta). Multiple alternatively spliced transcript variants encoding different isoforms have been identified. A representative sequence for the human CD74 gene is provided in Gene ID: 972 (see also sembl:ENSG00000019582, MIM:142790 and Vega:OTTHUMG00000163559).

One aspect of the invention provides pharmaceutical preparations that inhibit or reduce the role in disease development or progression of IBD stem cells or their progeny that have upregulated expression of CD74, i.e., to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease. These include, with limitation, nucleic acids, peptides and polypeptides (including antibodies), natural products, small molecules and carbohydrates. In certain embodiments, the therapeutic agent is locally delivered to the area of the gut afflicted by IBD, such as by oral delivery or local administered by injection or surgical placement.

CD74 interacts with MHC class I and II proteins, contributing to antigen presentation. CD74 directs transport of MHC class II α and β chains from the endoplasmic reticulum (ER) or the cell surface to endosomes. As a chaperone, CD74 contributes to peptide editing in the MHC class II compartment. In endosomes, proteases degrade CD74, releasing MHC class II molecules. Prevention of CD74 degradation promotes the cell surface localization of MHC II. In certain embodiments, the invention targets the role of CD74 in MHC Class II trafficking and antigen presentation by IBD stem cells and IBD tissues by reducing the expression of the protein in IBD stem cells and their progeny and/or enhancing the degradation of CD74.

CD74 on the cell surface also serves as a receptor for macrophage migration inhibitory factor (MIF) and d-dopachrome tautomerase (d-DT/MIF-2). In certain embodiments, the invention targets the role of CD74 a cell surface receptor, such as by reducing the expression of the protein in IBD stem cells and their progeny, or inhibiting the interaction of CD74 with MIF and/or d-DT/MIF-2 such as through agents which bind the receptor and block either binding or signaling by MIF and/or d-DT/MIF-2 (such as antibodies and ligand antagonists) or receptor decoys or other agents which bind MIF and/or d-DT/MIF-2 and prevent their interaction with CD74.

In certain embodiments, the invention provides pharmaceutical preparations of nucleic acids that inhibit or reduce the expression of CD74 to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease. These include, with limitation, antisense nucleic acids or RNA intereference nucleic acids (such as siRNA, miRNA and shRNA) or a sequence-directed ribozyme which include sequences that hybridize to a portion of the CD74 gene sequence or RNA transcript therefrom.

In certain embodiments, the invention provides pharmaceutical preparations of antibodies that selectively bind to and inhibit or reduce the biological function of CD74 to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease.

In certain embodiments, the invention provides pharmaceutical preparations of antibody-drug conjugates (ADC) including antibodies that selectively bind to CD74 to be used as part of a treatment protocol for patients having or at risk of developing IBD,

particularly Crohn's disease. The targeting of CD74 with the antibody portion of the ADC permits the delivery of a therapeutic agent to the diseased portion of the gut. In certain embodiments, the drug component of the ADC is cytotoxic or cytostatic to the cell expressing the CD74 protein to which the ADC binds. In other embodiments, the drug component of the ADC inhibits the differentiation and/or epigenetic traits of the the cell expressing the CD74 protein to which the ADC binds.

Another aspect of the invention provides diagnostic reagents for detecting the upregulated expression of CD74, for example, to identify the presence or absence of IBD stem cells in a biopsy or to image IBD impacted portions of the gut. These include, with limitation, nucleic acids and CD74 binding agents suchas antibodies.

o. SERPINB2 Agents

Given the pronounced overexpression of SERPINB2 in the isolated stem cells of pediatric Crohn's patients, another aspect of the invention relates to the use and pharmaceutical preparations of agents which reduce the expression of the SERPINB2 gene or reduce or inhibit the biological activity of the SERPINB2 protein, as as well as the detection of SERPINB2 gene expression and SERPINB2 protein levels as part of diagnostic assays and imaging protocols for identifying the presence of Crohn's stem cells or their progeny in patient samples or in in vivo imaging.

The SERPINB2 gene is a memebr of the the serpin family of proteins characterized by a unique tertiary structure and, unlike standard mechanism inhibitors, employ a suicide-substrate-like mechanism to neutralize their target proteinases. SerpinB2 (also called plasminogen activator inhibitor 2; PAI-2), has been implicated in the negative regulation of caspase-1.In fact, expression of SerpinB2 in macrophages deficient in NF-κB signalling, blocked their spontaneous IL-1β secretion, indicating that this serpin might serve as a negative regulator of caspase-1 and its upregulation in IBD may be part of the mechanism underlying increased susceptibility to microbial infections in IBD patients. Indeed, the most highly expressed gene commonly elevated in both Crohn's and UC is SerpinB2. A representative sequence for the human SERPINB2 gene is provided in Gene ID: 5055 (see also Esembl:ENSG00000019582, MIM:142790 and Vega:OTTHUMG00000163559).

Native, metastable serpins such as serpinB2 inherently tend to undergo stabilizing conformational transitions. This intrinsic tendency is modifiable by ligand binding, thus structure-based drug design is an attractive strategy for developing inhibitors of SerpinB2 (i.e., an inhibitor that prevents the inhibitory activity of serpinB2) such as small molecules, aptamers, antibodies and the like.

One aspect of the invention provides pharmaceutical preparations that inhibit or reduce the role in disease development or progression of IBD stem cells or their progeny

that have upregulated expression of SERPINB2, i.e., to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease. These include, with limitation, nucleic acids, peptides and polypeptides (including antibodies), natural products, small molecules and carbohydrates. In certain embodiments, the therapeutic agent is locally delivered to the area of the gut afflicted by IBD, such as by oral delivery or local administered by injection or surgical placement.

In certain embodiments, the invention provides pharmaceutical preparations of nucleic acids that inhibit or reduce the expression of SERPINB2 to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease. These include, with limitation, antisense nucleic acids or RNA intereference nucleic acids (such as siRNA, miRNA and shRNA) or a sequence-directed ribozyme which include sequences that hybridize to a portion of the SERPINB2 gene sequence or RNA transcript therefrom.

In certain embodiments, the invention provides pharmaceutical preparations of antibodies that selectively bind to and inhibit or reduce the biological function of SERPINB2 to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease.

Another aspect of the invention provides diagnostic reagents for detecting the upregulated expression of SERPINB2, for example, to identify the presence or absence of IBD stem cells in a biopsy or to image IBD impacted portions of the gut. These include, with limitation, nucleic acids and SERPINB2 binding agents suchas antibodies.

p. DPCR1 Agents

Given the pronounced overexpression of DPCR1 (diffuse panbronchiolitis critical region 1) in the isolated stem cells of pediatric Crohn's patients, another aspect of the invention relates to the use and pharmaceutical preparations of agents which reduce the expression of the DPCR1 gene or reduce or inhibit the biological activity of the DPCR1 protein, as as well as the detection of DPCR1 gene expression and DPCR1 protein levels as part of diagnostic assays and imaging protocols for identifying the presence of Crohn's stem cells or their progeny in patient samples or in in vivo imaging.

The DPCR1 gene is located between HLA-B and HLA-A on chromosome 6p21.33, is classified as one of the MHC class I molecules. The deduced 235-amino acid protein contains an N-terminal domain of about 164 amino acids that shares significant homology with the mucin-like repeat domain of zonadhesin (ZAN; 602372), followed by a transmembrane domain and an intracellular C-terminal domain of 48 amino acids. A representative sequence for the human DPCR1 gene is provided in Gene ID: 135656 (see also Ensembl:ENSG00000168631, MIM:613928 and Vega:OTTHUMG00000031104).

One aspect of the invention provides pharmaceutical preparations that inhibit or reduce the role in disease development or progression of IBD stem cells or their progeny that have upregulated expression of DPCR1, i.e., to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease. These include, with limitation, nucleic acids, peptides and polypeptides (including antibodies), natural products, small molecules and carbohydrates. In certain embodiments, the therapeutic agent is locally delivered to the area of the gut afflicted by IBD, such as by oral delivery or local administered by injection or surgical placement.

In certain embodiments, the invention provides pharmaceutical preparations of nucleic acids that inhibit or reduce the expression of DPCR1 to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease. These include, with limitation, antisense nucleic acids or RNA intereference nucleic acids (such as siRNA, miRNA and shRNA) or a sequence-directed ribozyme which include sequences that hybridize to a portion of the DPCR1 gene sequence or RNA transcript therefrom.

In certain embodiments, the invention provides pharmaceutical preparations of antibodies that selectively bind to and inhibit or reduce the biological function of DPCR1 to be used as part of a treatment protocol for patients having or at risk of developing IBD, particularly Crohn's disease.

Another aspect of the invention provides diagnostic reagents for detecting the upregulated expression of DPCR1, for example, to identify the presence or absence of IBD stem cells in a biopsy or to image IBD impacted portions of the gut. These include, with limitation, nucleic acids and DPCR1 binding agents suchas antibodies.

Other features and advantages of the invention will be understood by reference to the detailed description and examples that follow.

IV. Examples

The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. One skilled in the art may develop equivalent means or reactants without the exercise of inventive capacity and without departing from the scope of the invention.

The present work sought to address mucosal barrier properties in Crohn's through the clonal analysis of mucosal stem cells derived from both pediatric cases and age-matched controls. Our findings reveal that unlike control cases, pediatric Crohn's patients harbor two separate and epigenetically stable populations of mucosal stem cells distinguished by an inflammatory gene signature. Superimposed on the inflammatory trait is

a differentiation defect that severely alters the mucosal barrier properties of the terminal ileum. And while we trace the immediate basis of this differentiation defect to the repressed expression of Atonal BHLH transcription factor 1 (ATOH1), a gene required for secretory cell development in the colon[20], this repression is only emblematic of a more sweeping, homeotic conversion of terminal ileum stem cells to those of the proximal gastrointestinal tract that in turn dictates both the inflammatory and differentiation defects of these cells. Lastly, this coexistence of "Crohn's" and "normal" stem cells at the terminal ileum of these patients is likely of significance for the natural history of this disease as well as the rational basis of new therapeutic strategies.

Stable inflammatory gene signature

We cloned and propagated mucosal stem cells from endoscopic biopsies of children newly diagnosed with Crohn's disease and age-matched controls using recently developed technology that maintains columnar epithelial stem cells in a highly immature "ground state"[21,22]. In brief, approximately 150-200 independent stem cell colonies were derived from 1 millimeter (mm) biopsies of the terminal ileum and carried forward in sterile culture for minimum of eight weeks as either "pools" of clones or as subcloned "pedigrees" derived from single cells from these pools before analysis (Figure 1a). These pedigrees consist of highly immature stem cells that can be differentiated to a *3-D* intestinal mucosa by exposure to an air-liquid interface (ALI; Figure 1a). Unsupervised clustering of whole genome expression profiles of terminal ileum stem cell pedigrees from three Crohn's cases (CD1, CD2, and CD3), an age-matched "functional" control lacking mucosal inflammation (FC1), and a 22-week fetal demise case (FT1) revealed a bimodal distribution of gene expression profiles with a *Normal* cluster dominated by control and fetal terminal ileum pedigrees, and a second, *Crohn's* cluster occupied by some or most of the stem cell pedigrees derived from Crohn's patients (Figure 1b). However, we also noted that one or more of the stem cell pedigrees derived from the Crohn's cases appeared in the *Normal* cluster of the Principal Component Analysis (PCA) of whole genome expression profiles. The *Crohn's* cluster differentially expressed approximately 800 genes (>1.5-fold, $p<0.05$) compared to the *Control* cluster (Figure 1c). Filtering these genes against known inflammatory gene sets yielded nearly 200 that populate innate immune and antigen presentation pathways previously linked to Crohn's (Figure 1d; Figure 7). The differentiation of stem cell pedigrees amplified the distinctions between the *Normal* and *Crohn's* clusters to with nearly 1,200 genes (>1.5-fold, $p<0.05$) evident by PCA of whole genome expression datasets (Figure 2C). As seen in the stem cells of the *Crohn's* cluster, the gene expression profiling of the ALI-differentiated terminal ileum stem cells revealed an enrichment of genes associated with inflammatory pathways involving antigen presentation, innate immune responses, cytokine signaling (Figure 2D).

Defective secretory cell differentiation

Terminal ileum stem cells from control patients differentiate in ALI cultures to yield a *3-D* mucosa dominated by MUC2-expressing goblet cells (Figure 3A). These control epithelia also displayed markers for enteroendocrine cells (chromogranin A; CHGA) and Paneth cells (defensin 6A; DEF6A); (Figure 3A). In contrast, the majority of stem cell pedigrees derived from the terminal ileum of pediatric cases CD1, CD2, and CD3 yielded *3-D* epithelia that lacked obvious goblet cells and correspondingly showed little or no expression of MUC2 (Figure 3B). In addition, these *in vitro*-derived epithelia showed little or no staining by antibodies to the enteroendocrine marker CHGA or the Paneth cell marker DEFA6 (Figure 3B). However, all of the stem cell pedigrees from patients CD1, CD2, or CD3 that binned with the *Normal* cluster differentiated to *3-D* epithelial with the typical manifest of goblet cells, Paneth cells, and enteroendocrine cells produced by terminal ileum stem cells of control patients (Figure 3G). In addition to the defects in secretory cell differentiation, these *in vitro*-generated Crohn's cluster epithelia also showed an aberrant distribution pattern of glycoprotein A33 (GPA33; Figure 3B), a protein that forms an integral part of the tight junction in the colon and whose engineered deletion in mice yields a chronic inflammatory phenotype of the gastrointestinal tract[23]. At the same time, the *3-D Crohn's* epithelia showed a high, ectopic expression of claudin 18 (CLDN18), a tight junction protein, and of V-set and immunoglobulin domain containing 1 (VSIG1), a junctional adhesion protein (Figure 3B). These expression patterns in terminal ileum epithelial are supported by a broader comparison of expression profiles of particular genes associated with goblet, Paneth, and endocrine cells, all of which were low relative to control terminal ileum (Figure 13). Thus *Crohn's* cluster stem cells of the terminal ileum from all three cases showed consistent abnormalities in both secretory cell differentiation and intercellular junctional assembly.

Homeotic transformation of *Crohn's* stem cells

In addition to an extensive inflammatory gene signature and defective maturation of secretory cells, *Crohn's* cluster epithelia were distinguished by the ectopic expression of a host of metabolic enzymes *that had no obvious links to either inflammation or secretory cell differentiation (Figure 15A).* We were struck by the fact that these enzymes, which function in the hydrolysis and transport of lipids, carbohydrates, and proteins, are normally expressed in proximal portions of gastrointestinal tract 12-14 feet anterior to the terminal ileum (Figures 4A, 4B and 15A)[24]. To understand the scale and form of this ectopic gene expression, we mapped the gene expression profiles of the *Crohn's* cluster terminal ileum stem cells against those of each region of the gastrointestinal tract and their corresponding differentiated

epithelia derived from a 22-week fetal demise case[21]. To do this we first identified and mapped all differentially expressed genes (1.8-fold, $p<0.05$) of normal fetal stem cells and their corresponding differentiated epithelia along the fetal gastrointestinal tract and then cross-indexed these datasets with all differentially expressed genes of the *Crohn's* and *Normal* clusters. This analysis yielded a set of 271 genes that were both differentially expressed between *Crohn's* and *Normal* epithelia and showed regional expression along the normal fetal gastrointestinal tract (Figure 4B). Pathway analysis of these genes showed the most significant categories to be related to the metabolism and transport of nutrients (Figure 4B). Mapping the genes over-represented in the *Crohn's* cluster terminal ileum epithelia to discrete regions of the fetal gastrointestinal tract revealed a shift in their distribution to one centered around gastric, duodenum and jejunum epithelia, whereas those over-represented in control terminal ileum generally mapped throughout the colon (Figures 4C and 4D). The distribution patterns were highly similar across CD1, CD2, and CD3. Taken together, these data suggest that the terminal ileum stem cells of the *Crohn's* cluster displayed a switch in gene expression reminiscent of those driven by "homeotic" mutations in flies, which alter the developmental fate of segments along the *Drosophila* anterior-posterior body axis[25,26]. In particular, loss-of-function mutations in *homeobox* genes of the *Bithorax* complex (*BX-C*) result in a shift in the identity of posterior thoracic and abdominal segments (T3 to A8) to more that of the more anterior, mesothoracic (T2) segment[25-27]. Similarly, loss-of-function mutations of the *Antennapedia* complex (*ANT-C*) result in transformations of more anterior thoracic segments to the posterior T2 segment[26]. As *homeobox*-containing genes located within the four discrete *HOX* loci in humans play roles in tissue identity somewhat analogous to those of the Drosophila *Bithorax* and *Antennapedia* complexes[28-31], we asked whether the alterations apparent in Crohn's terminal ileum stem cells were accompanied by changes in the epigenetic profiles of these loci. Using whole-genome analyses of epigenetic histone marks, we identified multiple alterations in the epigenetic profiles of the *HOX* loci of stem cells of the *Crohn's* cluster compared to those of the *Normal* cluster (Figure 16A). In particular, both the *HOXA* and *HOXB* loci in *Crohn's* cluster stem cells showed extensive regions of repressive histone 3 lysine 27 trimethylation (H3K27me3) at their 5' ends (Figure 16A; Figure 16C), a finding paralleled by the repression of HoxA13 and HoxA11 transcripts compared to normal terminal ileum stem cells (Figure 16C). In contrast, the 3' portions of the *HOXA, B,* and *C* loci of Crohn's cluster stem cells showed a pattern of histone marks consistent with an overall potentiation or activation of 3' *HOX* genes relative to the *Normal* cluster stem cells (Figure 16A; Figure 16C). Interpreted in the context of prior studies of the patterning functions of the *HOX* loci[28-31], these data reveal a general epigenetic repression of genes situated at the 5'-ends of the *HOXA* and *HOXB* involved in posterior patterning, and a derepression of genes located at the 3'-ends of these loci generally linked to anterior fates.

As such, these findings are consistent with the shift in gene expression profiles of *Crohn's* cluster stem cells derived from the terminal ileum to patterns of proximal gastrointestinal tract. In addition to epigenetic alterations to the *HOX* loci, we found that more than 50% of the differentially expressed genes between *Crohn's* and *Normal* cluster stem cells had histone modifications with a bias for H3K4 trimethylation and H3K27 demethylation for over-represented transcripts and a de-acetylation of H3K27 at genes of relatively low expression. Among these genes are a host of other transcription factors including *CDX2* and *GATA5*, whose respective roles distal and proximal gastrointestinal tract differentiation are well established[32,33] (Figure 16B).

Inflammatory signature driven by homeotic shift

Given the consistency of the traits of inflammation, secretory cell defects, and homeotic transformations of terminal ileum stem cells across the Crohn's cases examined, we wanted to determine whether they were somehow interdependent. We first asked whether the inflammatory gene signatures of *Crohn's* cluster stem cells followed the overall shift to proximal gastrointestinal tract gene expression seen in the *Crohn's* cluster stem cells. Our mapping of genes of the inflammatory signature across those differentially expressed in the fetal gastrointestinal tract revealed a similar shift in genes overexpressed in Crohn's disease to more anterior regions of the gastrointestinal tract whereas those under-expressed mapped to the fetal colon. In fact, approximately 80% of the genes over-represented in the inflammatory signature are normally expressed in proximal (gastric, duodenum, jejunum) portions of the fetal gastrointestinal tract but not in the colon. Conversely, 67% of genes under-represented in the inflammatory signature are normally expressed in the colon of the fetal gastrointestinal tract. A sampling of genes whose over- (e.g. AHR, IL1RN, MGAT5, SMAD3, FUT2, and DUOX2) and under- (e.g. CLCA1, GUCY2C, IL37, and NOX1) representation might be a consequence of the homeotic transformation underscores the potential immunoregulatory impact of such a conversion (Figure 19A). Consistent with the stability of the inflammatory signature in the Crohn's stem cells, 71% of the 180 genes showing enhanced expression in the inflammatory signature also had differential histone modifications marked by increased H3K4-trimethylation and a loss of H3K27-trimethylation typical of active or potentially active genes. Of down-regulated genes in the inflammatory gene signature, 48% had differential histone modifications and these were dominated by a loss of H3K27-acetylation and a gain of H3K27-trimethylation associated with gene repression (Figure 17A). Examples of such over- and under-represented genes with histone modifications include the transcription factor aryl hydrocarbon receptor (AHR) and the enterotoxin receptor GUCY2C (Figure 17B).

Our analysis of the inflammatory gene signatures of the Crohn's-derived stem cells revealed the differential expression of multiple genes (e.g. AHR, SMAD3, CCL2, FUT2) implicated by GWAS. To examine more broadly the relationship between the *Crohn's* cluster stem cells and the genetic architecture of Crohn's, we compared the set of genes linked by GWAS to Crohn's from multiple studies[7-10] with those differentially expressed genes by *Crohn's* cluster stem cells and the epithelia generated from them and those predicted by disease association algorithms (e.g. *GRAIL*). A majority of the matches identified single genes among the multiple genes within a linkage disequilibrium (LD) block (e.g. NUPR1 at rs26526), and several highlight multiple genes coordinately upregulated within an LD block containing related genes such as CXCL1, 2, 3, and 5 at rs2472649[8], and class II MHC genes HLA-DRB1 and HLA-DRA at rs6927022[8]. We found an overlap of 28% of genes implicated by GRAIL and those differentially expressed by the Crohn's cluster cells (Figure 19B), as will as another set of genes that were not captured by GRAIL and whose significance at this point is unclear. Regardless, the overlap between genes implicated by GWAS and those differentially expressed in Crohn's disease epithelia suggests broad links between these mucosal stem cells and the disease process.

Homeotic shift underlying differentiation defects

ATOH1 is known to regulate secretory cell differentiation in the airways and colon via its control of a network of downstream transcription factors that includes SPDEF, GFI1, and NEUROG3[20,34-37] (Figure 4F). Given the impact of the homeotic shift of *Crohn's* cluster terminal ileum stem cells on inflammatory gene expression, we asked whether the transcription factors regulating secretory cell differentiation were similarly affected by this shift. Mapping the expression profiles of ATOH1, SPDEF, GFI1, and NEUROG3 onto the regiospecific expression patterns in the human fetal intestine showed that all are highly expressed in the distal gastrointestinal tract but much less so in proximal segments (Figure 4F). Consistently, ATOH1 expression was differentially low in terminal ileum stem cells of the *Crohn's* cluster compared with those of the *Normal* cluster (Figure 5A). We therefore asked if we could rescue the secretory cell phenotype of the Crohn's disease terminal ileum stem cells through supplementing ATOH1 expression *via* retroviral transduction. We transduced *Crohn's* cluster stem cells with a retrovirus driving ATOH1 and GFP expression and subsequently differentiated them in ALI cultures (Figure 5B, Figure 5E). This maturation of ATOH1-transduced cells yielded an epithelium replete with MUC2-expressing goblet cells. Expression profiling of goblet, Paneth, and endocrine cell markers showed that the virally-driven expression of ATOH1 resulted in a induction of genes associated with goblet cells and Paneth cells, though the impact on enteroendocrine cells was less obvious (Figure 5F). Despite ATOH1's ability to complement the secretory cell defect in Crohn's cluster stem

cells, these rescued cells showed only minor changes in gene expression generally involving a handful of secretory cell genes. Moreover, the rescue of this secretory cell defect did not appreciably alter the overall pattern of differential gene expression underlying the homeotic shift in the terminal ileum (Figure 5H, Figure 5I). We also performed the reverse experiment to assess the effect of the bi-allelic disruption[38] of ATOH1 in normal terminal ileum stem cells on their differentiation potential (Figure 5J). Compared to the extreme differentiation defect seen in the Crohn's terminal ileum stem cells, the bi-allelic disruption of ATOH1 in normal cells showed a hypomorphic pattern of differentiation of secretory cells (Figure 5L). Apart from this partial phenocopying of the Crohn's differentiation defect, our analysis of whole genome expression data from ATOH1-null normal terminal ileum stem cells showed no evidence for the induction of either an inflammatory gene signature or of the more general homeotic shift in gene expression.

Coexistence of normal and Crohn's stem cells

The CD3 case was unusual in that the single terminal ileum biopsy yielded roughly equal numbers of stem cells in the *Crohn's* or *Normal* cluster whereas CD1 and CD2 were dominated by stem cells of the *Crohn's* cluster (c.f. Figure 1). Using antibodies against cell surface markers expressed by *Crohn's* cluster stem cells such as VSIG1, we could readily identify and select stem cell colonies from the CD3 pool of clones that have the *Crohn's* or *Normal* traits (Figure 6A and 6B). Pedigrees derived from VSIG1-negative colonies differentiated in ALI culture to terminal ileum with a normal complement of secretory cells, whereas those from VSIG1-positive pedigrees differentiated to epithelia with the typical secretory cell defects (Figure 6B). The gene expression profiles of these two types of pedigrees also clustered by PCA and with their respective counterparts from CF1, CD1, and CD2 terminal ileum at both the stem cell and ALI differentiation levels (Figure 6D). The characteristics of these two populations of stem cells from the CD3 terminal ileum seemed absolute in that those in the *Crohn's* cluster had the inflammatory gene signature, secretory cell defects, and the homeotic transformation whereas those of the *Normal* cluster had none of these features. Based on these findings, we screened larger numbers of colonies from the CD1 and CD2 terminal ileum biopsies and identified colonies equivalent to those of the *Normal* cluster in each of them. These findings provide strong evidence for the coexistence of normal and Crohn's disease stem cells in close proximity in the same patient and may underlie the observed, more macroscopic "skip-lesion" patterning that is a hallmark of Crohn's disease colonic mucosa[1]. The binary properties of these stem cells might also explain the lack of established histopathology correlates in the terminal ileum of Crohn's disease patients that might be expected if *all* terminal ileum stem cells were uniform for the secretory cell differentiation defects observed in this study. Nevertheless, we asked whether

antibodies specific to the epithelia generated *in vitro* from the *Crohn's* cluster stem cells could recognize discrete patterns in histological sections of Crohn's and normal biopsies. Our analysis reveal the co-mingling of normal epithelial glands dominated by MUC2-positive goblet cells and glands devoid of obvious goblet cells that reacted strongly with antibodies to VSIG1, PRSS2, and CLDN18, all gene products overexpressed by epithelia derived from cloned stem cells of the *Crohn's* cluster. None of these atypical foci were evident in control sections. We note that our findings are reminiscent of observations of 'pyloric gland" or "gastric" metaplasia in mucosal histological sections of Crohn's patients first described more than 60 years ago whose significance for the dynamics of Crohn's disease has remained unclear[39-41].

Discussion

The key finding of this work is that the terminal ileum of pediatric patients with Crohn's disease harbors both normal and abnormal stem cells. The variant stem cells, as well as epithelia derived from them, show highly stable gene expression profiles tied to aberrant processes of inflammation, mucosal barrier formation, and metabolic processes more typical of epithelia found more in proximal portions of the gastrointestinal tract. About a third of these differentially expressed genes are linked to inflammatory pathways of the innate immune response, antigen presentation, and interferon signaling previously implicated in Crohn's disease. In addition, the overall gene expression profiles of these cells overlap with those found in 28% of GWAS loci described for Crohn's disease, suggesting a proximity between these stem cells and the pathology of Crohn's. While the relative contributions of mucosal stem cells and cells of the adaptive and innate immune system[18] to Crohn's disease pathogenesis remains unclear, the chronic nature of the inflammatory signaling in seen in this aberrant population of stem cells may impart to them disproportionate influence. If so, efforts to interfere with nodal aspects of their inflammatory gene signatures could do much to limit the morbidity of this disease.

Beyond their inflammatory character, the terminal ileum stem cells of all three cases showed a severe, cell-autonomous defect in secretory cell differentiation that underscores and extends the barrier defect hypothesis in this disorder[42]. Our findings confirm earlier genetic and pathophysiology data implicating mucosal and specifically Paneth cells defects[11,,16,17], and expand the secretory cell phenotype in the terminal ileum to include defective differentiation of goblet and enteroendocrine cells. In light of their contributions to the mucus layer, enteric nervous system control[43], and anti-microbial factors, the combined loss of these secretory cells could render the terminal ileum particularly vulnerable to microbial challenge.

The most exotic and likely pathogenic feature of the terminal ileum stem cells of the *Crohn's* cluster is the shift in overall gene expression to that of an amalgam of proximal gastrointestinal epithelia including those of the stomach, duodenum and jejunum. As with the inflammatory gene signature and the defective secretory cell differentiation, this state of homeotic transformation was stable and stereotyped across cases and across at least six months of continuous growth in culture. And while the expression of metabolic enzymes typical of the proximal gastrointestinal tract was an initial hint of this homeotic transformation, our epistasis analyses support the notion that the homeotic transformation underlies both the inflammatory gene signature and the defective secretory differentiation of the *Crohn's* cluster stem cells. Thus the enhanced expression of genes such as AHR, MGAT5, SMAD3, DUOX2 and the loss of others such as GUCY2C, HLA-C, ALOX5, and NOX1 in *Crohn's* cluster stem cells is consistent with the shift in expression patterns of these genes between the terminal ileum and those of proximal gastrointestinal epithelia. Similarly, this homeotic transformation simultaneously represses ATOH1 and other transcription factors including SPDEF, GFI1, and NEUROG3 involved in secretory cell differentiation which likely explains why the differentiation defect is so much more extreme in the *Crohn's* cluster stem cells than those of the *Normal* cluster engineered for a biallelic disruption of ATOH1 alone. In a minimal sense the homeotically transformed terminal ileum would acquire barrier, immunological, and metabolic properties of proximal gastrointestinal tract devoted to nutrient processing and absorption in the near absence of gut microbes. Consequently, the transformed epithelial would be ill-suited to manage the 10,000-fold increase in intestinal microbes at the terminal ileum *versus* normal duodenum[44]. Experimentally, patterning defects due to homeotic transformations have been induced in flies or mammals by mutations of the *Bithorax* complex or homologous *HOX* loci, respectively[28-31]. It will be critical to understand how epigenetic mechanisms[45,46] might cooperate with environmental influences and genetic predisposition to bring about such transformations. Finally, the coexistence of two populations of stem cells in terminal ileum of pediatric Crohn's patients is physically manifested by the simultaneous presence of normal and metaplastic glands in the terminal ileum biopsies and resections examined here and in previous studies of Crohn's and inflammatory bowel disease in general[47-50]. If indeed these cells and their associated metaplasia contribute to Crohn's, their existence and specifically their coexistence with normal mucosal stem cells could inform new therapeutic strategies directed at neutralizing their pathogenic impact, reprogramming them to normalcy, or their selective eradication altogether.

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Methods

***In vitro* culture of human terminal ileum and colonic epithelial stem cells**

Terminal ileum and right colon endoscopic biopsies were obtained from newly diagnosed pediatric Crohn's patients and functional controls lacking mucosal inflammation under informed parental consent and institutional review board approval at the Connecticut Children's Medical Center, Hartford, CT USA and the University of North Carolina, Chapel Hill, Chapel Hill, NC, USA. 1 mm endoscopic biopsies were collected into cold F12 media (Gibco, USA) with 5% fetal bovine serum (Hyclone, USA), and then were minced by sterile scalpel into 0.2-0.5 mm^3 sizes with a viscous and homogeneous appearance. The minced tissue was digested in 2 mg/ml collagenase type IV (Gibco, USA) at 37℃ for 30-60 min with agitation. Dissociated cells were passed through a 70 µm Nylon mesh (Falcon, USA) to remove masses and then were washed four times in cold F12 media, and seeded onto a feeder layer of lethally irradiated 3T3-J2 cells in cFAD media containing 125 ng/mL R-Spondin1 (R&D systems, USA), 1 µM Jagged-1 (AnaSpec Inc, USA), 100 ng/ml Human

Noggin (Peprotech, USA), 2.5 µM Rock-inhibitor (Calbiochem, USA), 2 µM SB431542 (Cayman chemical, USA), 10 mM Nicotinamide (Sigma-Aldrich, USA). Cells were cultured at 37℃ in a 7.5% CO_2 incubator. The culture media was changed every two days. Colonies were digested by 0.25 % trypsin-EDTA solution (Gibco, USA) for 5-8 min and passaged every 7 to 10 days. Colonies were trypsinized by TrypLE Express solution (Gibco, USA) for 8-15 min at 37℃ and cell suspensions were passed through 30 µm filters (Miltenyi Biotec, Germany). Approximately 20,000 epithelial cells were seeded to each well of 6-well plate. Cloning cylinder (Pyrex, USA) and high vacuum grease (Dow Corning, USA) were used to select single colonies for pedigrees. Gene expression analyses were performed on cells derived from passage 4-10 (P4-P10) cultures, at which the GI tract derived stem cells were observed to be both genetically and epigenetically stable and for all detectable purposes identical to the passage 1 stem cells from clone from which each was derived.

Histology and Immunostaining

Histology, hematoxylin and eosin (H&E) staining, immunohistochemistry, and immunofluorescence were performed using standard techniques. For immunofluorescence and immunohistochemistry, 4% paraformaldehyde-fixed, paraffin embedded tissue slides were subjected to antigen retrieval in citrate buffer (pH 6.0, Sigma-Aldrich, USA) at 120 ℃ for 20 min, and a blocking procedure was performed with 5% bovine serum albumin (BSA, Sigma-Aldrich, USA) and 0.05 % Triton X-100 (Sigma-Aldrich, USA) in DPBS(-) (Gibco, USA) at room temperature for 1 hr. All images were captured by using the Inverted Eclipse Ti-Series (Nikon, Japan) microscope with Lumencor SOLA light engine and Andor Technology Clara Interline CCD camera and NIS-Elements Advanced Research v.4.13 software (Nikon, Japan) or LSM 780 confocal microscope (Carl Zeiss, Germany) with LSM software. Bright field cell culture images were obtained on an Eclipse TS100 microscope (Nikon, Japan) with Digital Sight DSFi1camera (Nikon, Japan) and NIS-Elements F3.0 software (Nikon, Japan).

Stem cell differentiation

Air-liquid interface (ALI) culture of terminal ileum and colonic epithelial cells was performed as described in the literature. Briefly, Transwell inserts (Corning Incorporated, USA) were coated with 20 % Matrigel (BD biosciences, USA) and incubated at 37 ℃ for 30 min to polymerize. 200,000 irradiated 3T3-J2 cells were seeded to each Transwell insert and incubated at 37 ℃, 7.5 % CO2 incubator overnight. QuadroMACS Starting Kit (LS) (Miltenyi Biotec, Germany) was used to purify the stem cells by removal of feeder cells. 200,000-300,000 stem cells were seeded into each Transwell insert and cultured with stem cell media. At confluency (3-7 days), the apical media on the inserts was removed through

careful pipetting and the cultures were continued in differentiation media (stem cell media without nicotinamide) for an additional 6-12 days prior to harvesting. The differentiation media was changed every one or two days.

RNA sample preparation

For stem cell colonies, RNA was isolated using PicoPure RNA Isolation Kit (Life Technologies, USA). For ALI structure, RNA was isolated using Trizol RNA Isolation Kit (Life Technologies, USA). RNA quality (RNA integrity number, RIN) was measured by analysis Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano Kit (Agilent Technologies, USA). RNAs having a RIN > 8 were used for microarray analysis.

Expression microarray and bioinformatics

Total RNAs obtained from immature colonies and ALI differentiated epithelia were used for microarray preparation with WT Pico RNA Amplification System V2 for amplification of DNA and Encore Biotin Module for fragmentation and biotin labeling (NuGEN Technologies, USA). All samples were prepared according to manufacturer's instructions and hybridized onto GeneChip Human Exon 1.0 ST or Human Transcriptome (HTA) Arrays (Affymetrix, USA). GeneChip operating software was used to process all the Cel files and calculate probe intensity values. To validate sample quality, quality checks were conducted using Affymetrix Expression Console software. The intensity values were log2-transformed and imported into the Partek Genomics Suite 6.6 (Partek Incorporated, USA). Exons were summarized to genes and a 1-way ANOVA was performed to identify differentially expressed genes. For two sample statistics, p-values were calculated by student t-test for each analysis. Unsupervised clustering and heatmap generation were performed with sorted datasets by Euclidean distance based on average linkage clustering, and Principal Component Analysis (PCA) map was conducted using all or selected probe sets by Partek Genomics Suite 6.6. Pathway analysese were performed with Gene Set Enrichment Analysis (GSEA) and Ingenuity Pathway Analysis (IPA) software.

Antibodies:

Mouse monoclonal Ki67 BD550609 (BD Pharmingen, USA)
Goat polyclonal E-Cadherin AF648 (R&D Systems, USA)
Goat polyclonal GFP ab5450 (Abcam, USA)
Rabbit polyclonal SOX9 ab5535 (Abcam, USA)
Rabbit polyclonal Chromogranin A (CHGA) ab15160 (Abcam, USA)
Rabbit polyclonal Glycoprotein A33 (GPA33) ab108938 (Abcam, USA)
Mouse monoclonal LI-Cadherin (Cdh17) sc-74209 (Santa Cruz, USA)

Rabbit polyclonal Mucin 2 (MUC2) sc-15334 (Santa Cruz, USA)

Rabbit polyclonal Claudin 18 (CLDN18) HPA018446 (Sigma-Aldrich, USA)

Rabbit polyclonal Defensin 6 (DEFA6) HPA019462 (Sigma-Aldrich, USA)

Rabbit polyclonal Mucin 17 (MUC17) HPA031634 (Sigma-Aldrich, USA)

Secondary antibodies:

Donkey anti-Rabbit IgG (H+L) AlexaFluor 594, A-21207 (ThermoFisher Scientific, USA)

Donkey anti-Goat IgG (H+L) AlexaFluor 488, A-11055 (ThermoFisher Scientific, USA)

Donkey anti-Mouse IgG (H+L) AlexaFluor 488, A-21202 (ThermoFisher Scientific, USA)

CLAIMS

1. A cell culture comprising a purified population of epithelial stem cells derived from gastrointestinal biopsies from IBD patients, wherein the isolation, passaging and maintenance of the subject IBD stem cells was carried out using a culture media system comprising a basal media and (a) a ROCK (Rho Kinase) inhibitor; (b) a Wnt agonist; (c); a Bone Morphogenetic Protein (BMP) antagonist; (d) a Notched inhibitor; (e) a TGFβ signaling pathway inhibitor (a TGFβ inhibitor or a TGFβ receptor inhibitor), and optionally may also include nicotinamide or an analog thereof and/or a mitogenic growth factor (EGF, KGF, TGFβ, BDNF, HGF, or bFGF), which cell culture conditions provide stable culture and passaging of the IBD stem cells under conditions that maintain the genotype and epigenetics of the stem cell as it existed in the biopsy.

2. A method of identifying a drug agent able to inhibit the growth or proliferation of an IBD stem cell and/or the generation of epithelial tissue from an IBD stem cell, comprising:
(i) contacting a preparation of IBD stem cells with a plurality of drug candidates;
(ii) identifying a drug candidate in step (i) that alters the IBD stem cells by one or more of inhibiting growth, proliferation or differentiation of the IBD stem cells and/or epigenetically modifies the IBD stem cells;
(iii) formulating the drug candidate identified in step (ii) in a pharmaceutical preparation suitable for administration to a human subject.

3. A method for treating or preventing an inflammatory bowel disease comprising treating a patient in need thereof with one or more agents that have one or more of the following effects: (i) reduction in the number of IBD stem cells in the patient; (ii) inhibit proliferation of the IBD stem cells; (iii) inhibit differentiation of the IBD stem cells; (iv) epigenetically modify the IBD stem cells to change the genotype and/or phenotype of cells and tissues generated from the IBD stem cells.

4. The method of claim 3, wherein the agent is selected from nucleic acids, peptides, polypeptides (including antibodies), natural products, small molecules and carbohydrates.

5. The method of claim 3 or 4, wherein the inflammatory bowel disease is Ulcertative Colitis.

6. The method of claim 3 or 4, wherein the inflammatory bowel disease is Corhn's Disease.

7. The method of claim 6, wherein the agent targets a pCD Gene Sequence or a protein encoded thereby.

8. The method of any of the preceding claims, wherein the agent is an antibody-drug conjugate.

9. A cell culture comprising a purified population of epithelial stem cells derived from gastrointestinal biopsies from IBD patients, which cell culture conditions provide stable culture and passaging of the stem cells under conditions that maintain the genotype and epigenetics of the stem cell as it existed in the biopsy.

10. A multiwell array of cell cultures comprising a plurality of individual wells each containing a purified population of epithelial stem cells derived from gastrointestinal biopsies from one or more IBD patients.

11. An antibody that selectively binds to a protein encoded by pCD Gene Sequence.

12. The antibody of claim 11, which is an antibody-drug conjugate.

13. Use of the antibody of claim 11 or 12 in the manufacture of medicament for the treatment of an inflammatory bowel disease.

14. A nucleic acid including a sequence that selectively hybridizes to a pCD Gene Sequence and inhibits expression of the pCD Gene Sequence to which it hybridizes.

15. Use of the nucleic acid of claim 14 in the manufacture of medicament for the treatment of an inflammatory bowel disease.

16. A kit for assessing a patient's risk of having or developing an inflammatory bowel disease, comprising (i) detection means for detecting the differential expression, relative to a normal cell, of one or more pCD Gene Sequences or the gene products thereof; and (ii) instructions for correlating the differential expression of pCD Gene Sequences or gene products, if any, with a patient's risk of having or developing an inflammatory bowel disease.

17. The kit of claim 16, wherein the detection means includes nucleic acid probes for detecting the level of mRNA of the one or more pCD Gene Sequences.

18. The kit of claim 16, wherein the detection means includes nucleic acid probes for detecting the presence of mutations or changes in methylation patterns to genomic sequences encoding the one or more pCD Gene Sequences.

19. The kit of claim 16, wherein the detection means includes an immunoassay for detecting the level of protein(s) encoded by the one or more pCD Gene Sequences.

20. Use of the cell cultures of claims 1 or 9 to identify genes or gene products for which the level of expression is altered by inflammatory bowel disease relative to normal healthy gastrointestinal tissue or the epigenetic pattern of the genome of the IBD stem cell is altered by inflammatory bowel disease relative to normal healthy gastrointestinal tissue.

21. Use of the cell cultures of claims 1 or 9 to identify drug agents that have one or more of the following effects: (i) reduction in the number of IBD stem cells in the patient; (ii) inhibit proliferation of the IBD stem cells; (iii) inhibit differentiation of the IBD stem cells; (iv) epigenetically modify the IBD stem cells to change the genotype and/or phenotype of cells and tissues generated from the IBD stem cells.

Fig. 1A

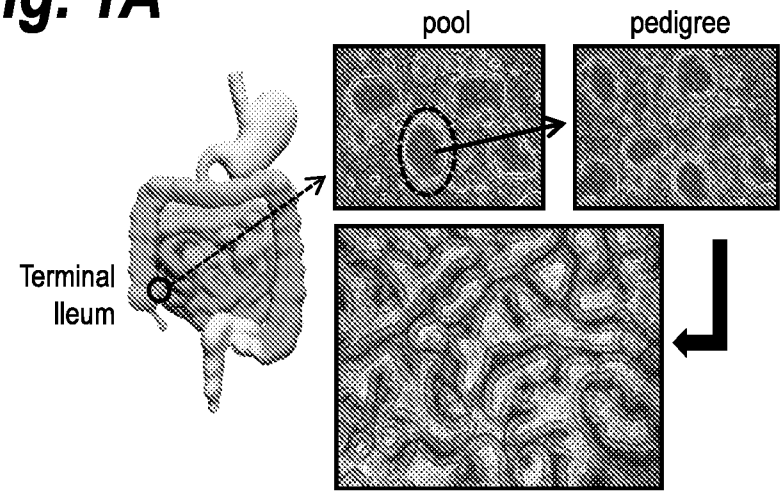

pool
pedigree
Terminal
Ileum

Fig. 1B

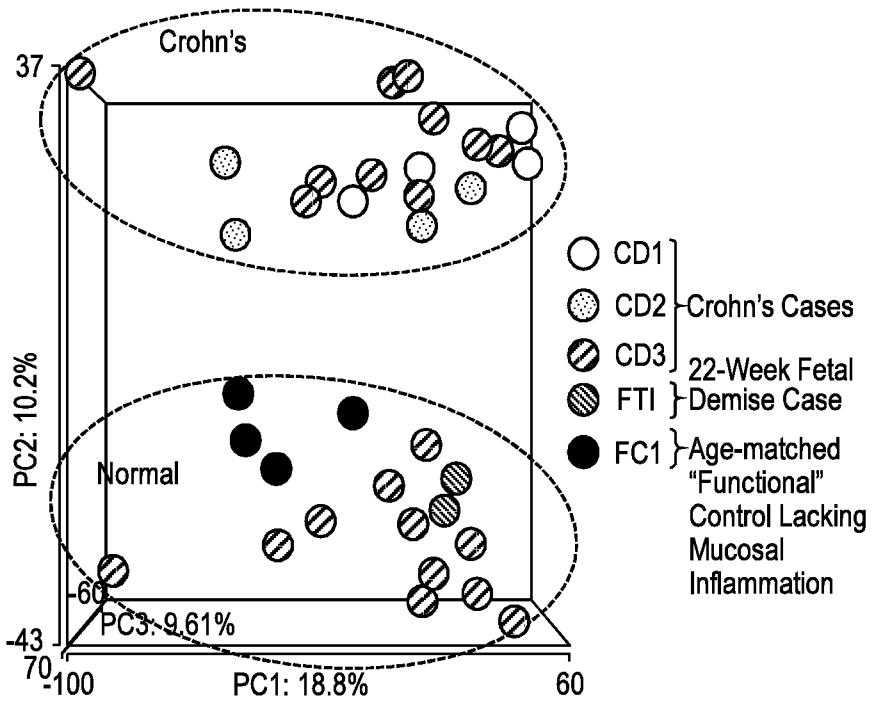
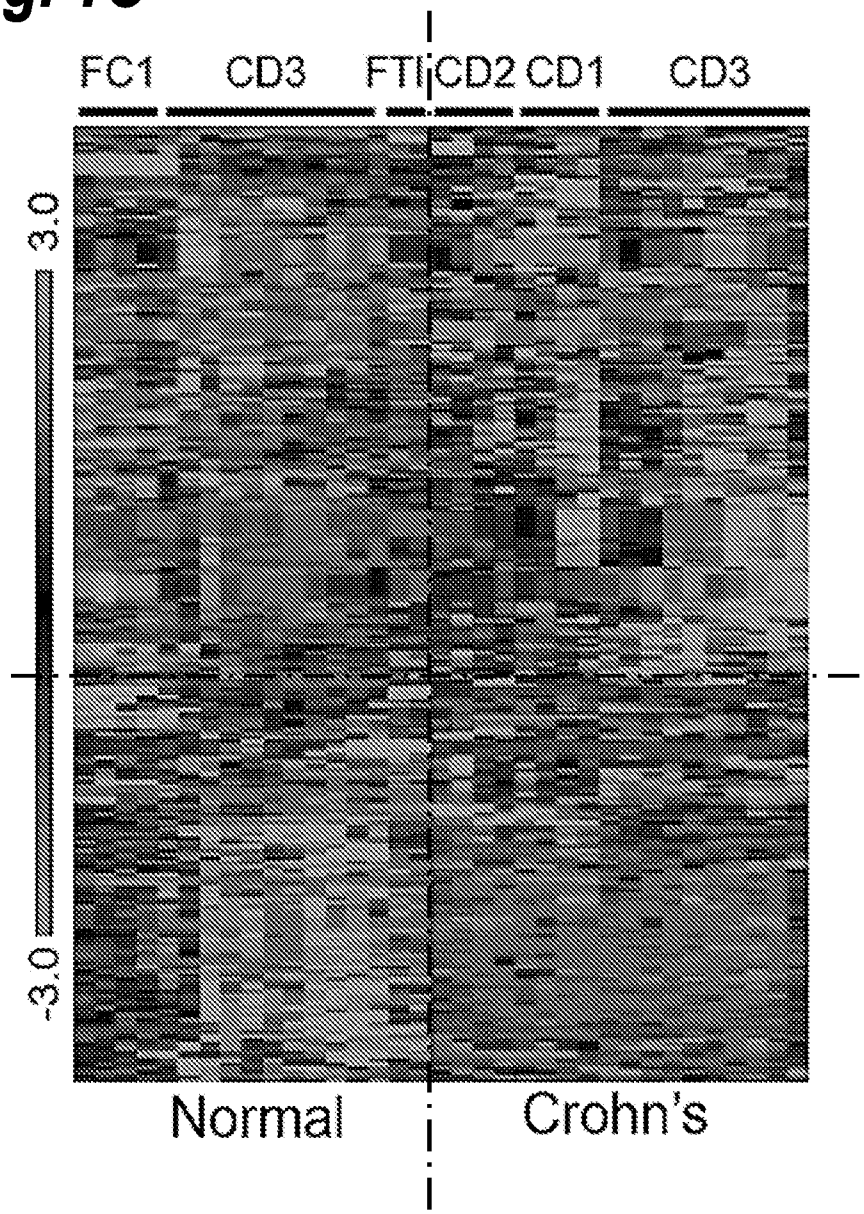
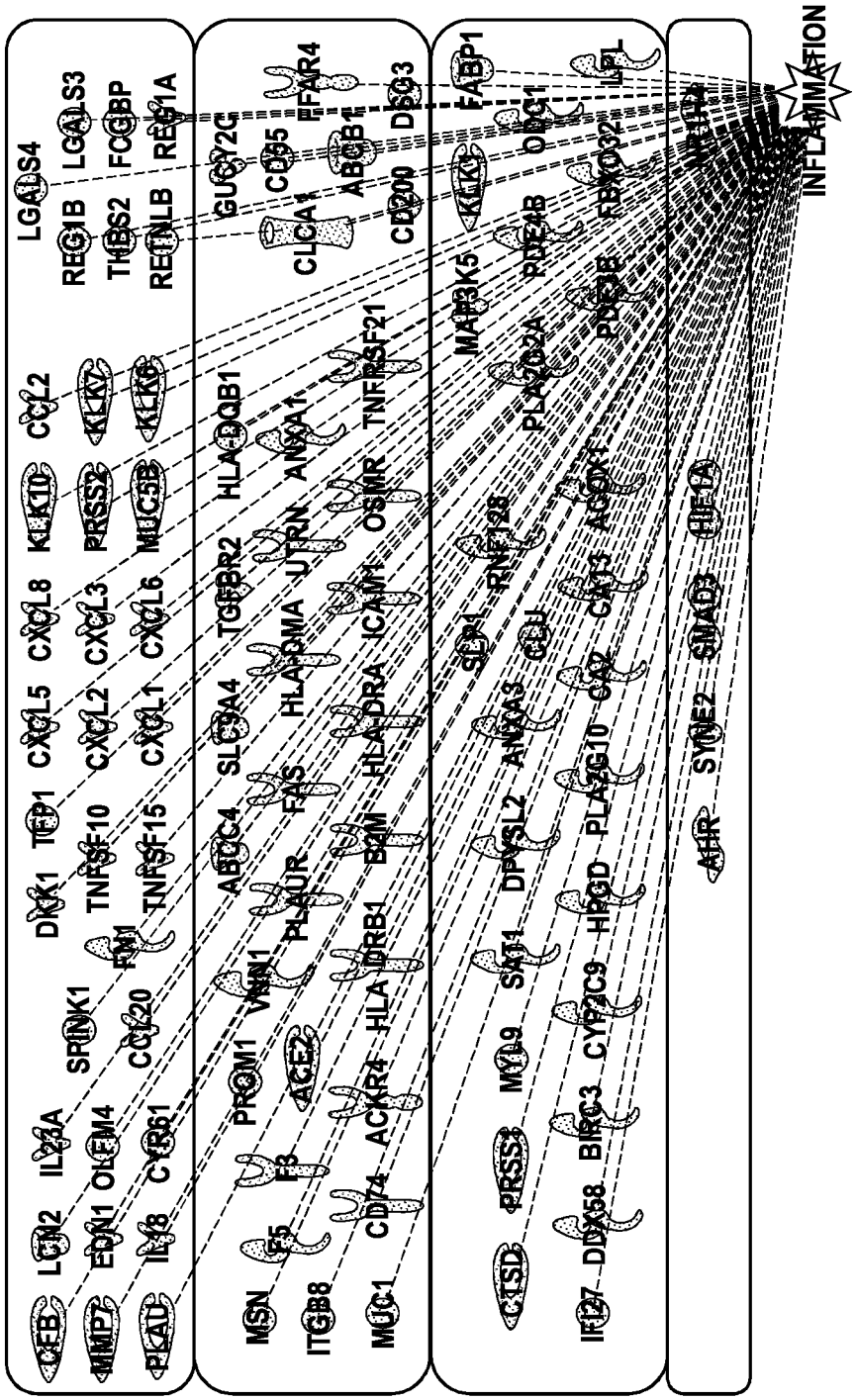

Crohn's
37
Normal
-60
-43
70
-100
PC1: 18.8%
60
PC2: 10.2%
PC3: 9.61%
CD1
CD2
CD3
Crohn's Cases
22-Week Fetal
FTI
Demise Case
FC1
Age-matched
"Functional"
Control Lacking
Mucosal
Inflammation

Fig. 1C

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LGALS3
THBS2
FCGBP
REG1LB
REG1A
GUCY2C
CD5
FFAR4
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CD200
DSG3
FABP1
KLK7
OLFM4
LPL
PDE4B
FBXO32
KLK6
MAPK5
HLA-DQB1
AMICA1
PRSS2
MUC5B
OSMR
TNFRSF21
CXCL6
UTRN
CXCL3
CXCL2
CXCL1
TGFBR2
CXCL5
HLA-DMA
CXCL8
SLC39A4
TCP1
ICAM1
KLK10
ABCC4
CCL2
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FAS
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PLAUR
CXCR1
HLA-DRB1
TNFRSF15
VNN1
FN1
ACKR4
CYR61
PROM1
SRPINK1
CD74
IL23A
F3
MUC1
ACE2
LCN2
F5
CEB
ITGB8
IL18
MSN
PLAU
MMP7
EDN1
CTSD
PRSS3
MYL9
SAT1
DPYSL2
ANXA3
CDU
SDR1
RNF128
PLA2G2A
ACOX1
PDE3B
CAT
GAS
PLA2G10
HPGD
CYP2C9
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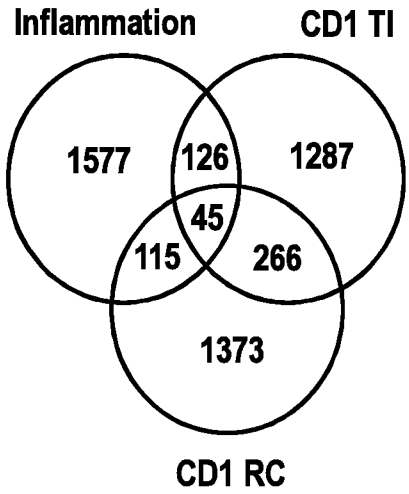
IFI27
AHR
SYNE2
SMAD3
HIF1A
INFLAMMATION

Fig. 1D

Fig. 1E



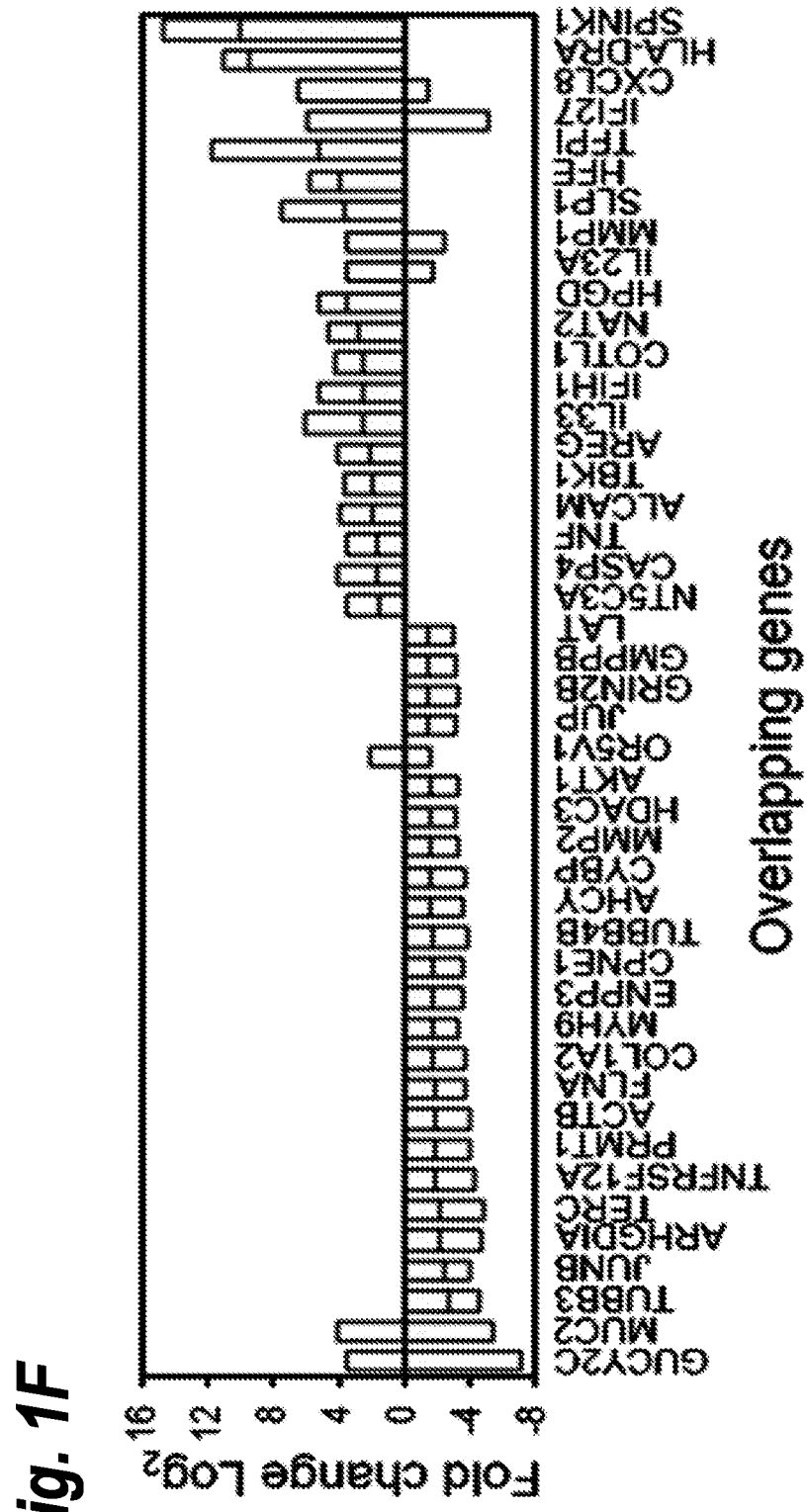

Fold change Log2
16
12
8
4
0
4
8
Overlapping genes
GUCY2C
MUC2
TUBB3
JUNB
ARHGDIA
TERC
TNFRSF12A
PRMT1
ACTB
FLNA
COL1A2
MYH9
ENPP3
CPNE1
TUBB4B
AHCY
CYBP
MMP2
HDAC3
AKT1
OR5V1
JUP
GRIN2B
GMPPB
LAT
NT5C3A
CASP4
TNF
ALCAM
TBK1
AREG
IL33
IFIH1
COTL1
NAT2
HPGD
IL23A
MMP1
SLP1
HFE
TFPI
IFI27
CXCL8
HLA-DRA
SPINK1

Fig. 1F

Fig. 2A

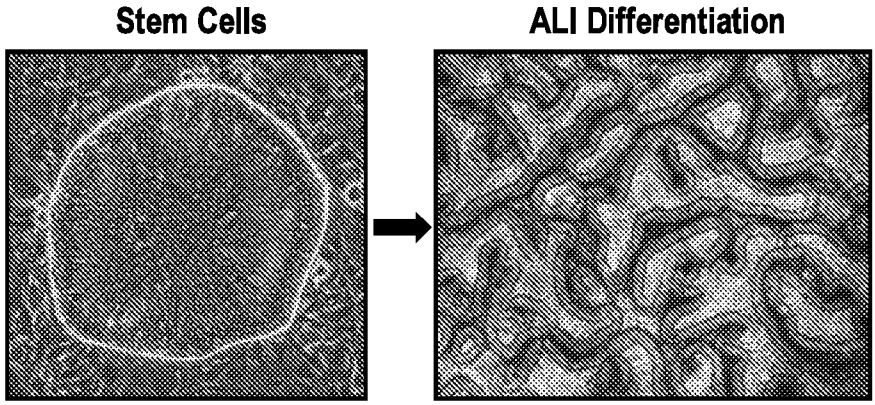
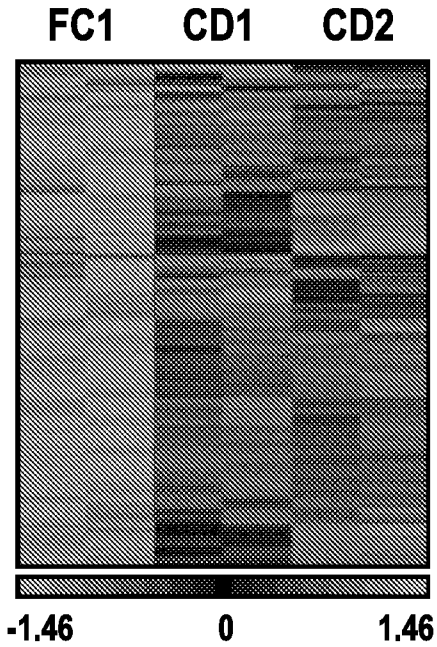


Fig. 2B



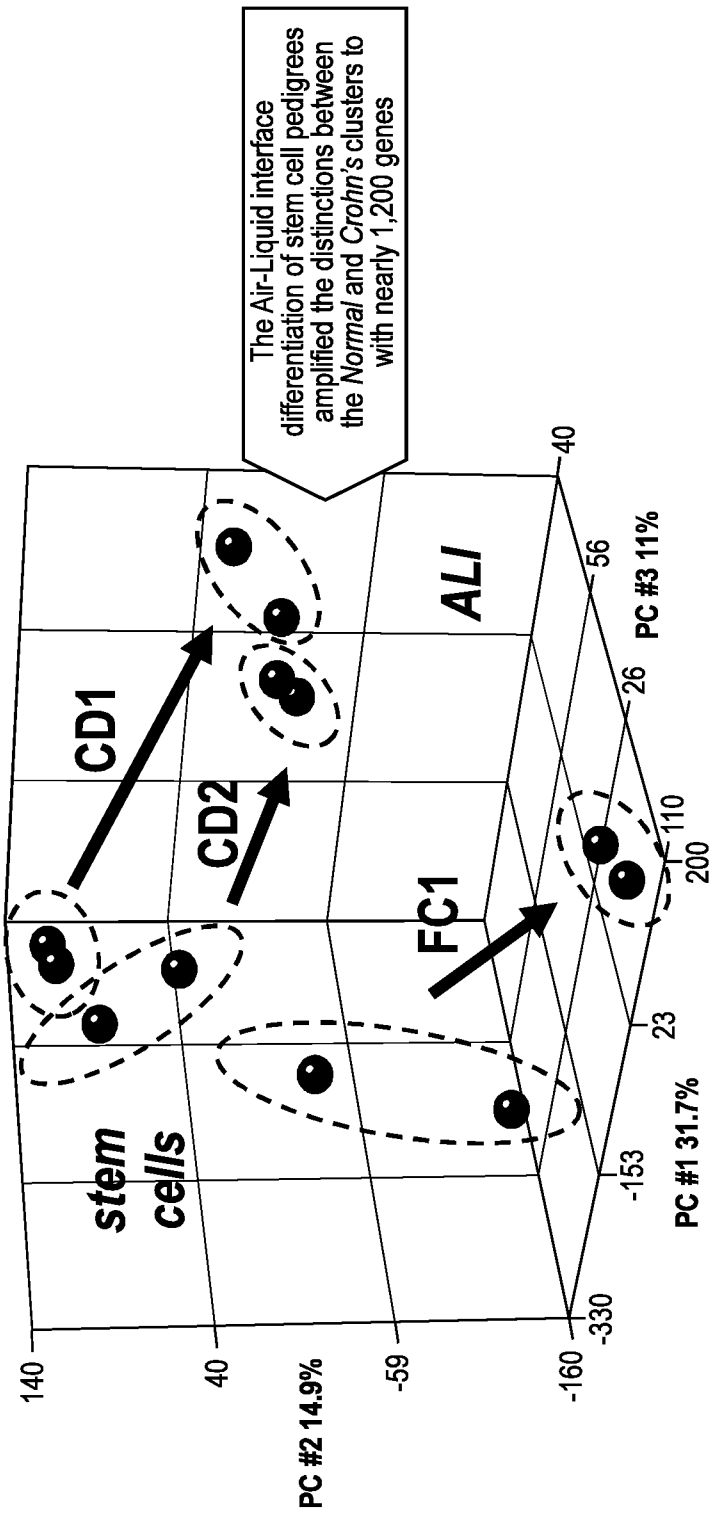

The Air-Liquid interface differentiation of stem cell pedigrees amplified the distinctions between the *Normal* and *Crohn's* clusters to with nearly 1,200 genes
stem cells
CD1
CD2
FC1
ALI
140
40
-59
-160
PC #2 14.9%
40
56
26
-3
-30
PC #3 11%
110
200
23
-153
-330
PC #1 31.7%

Fig. 2C

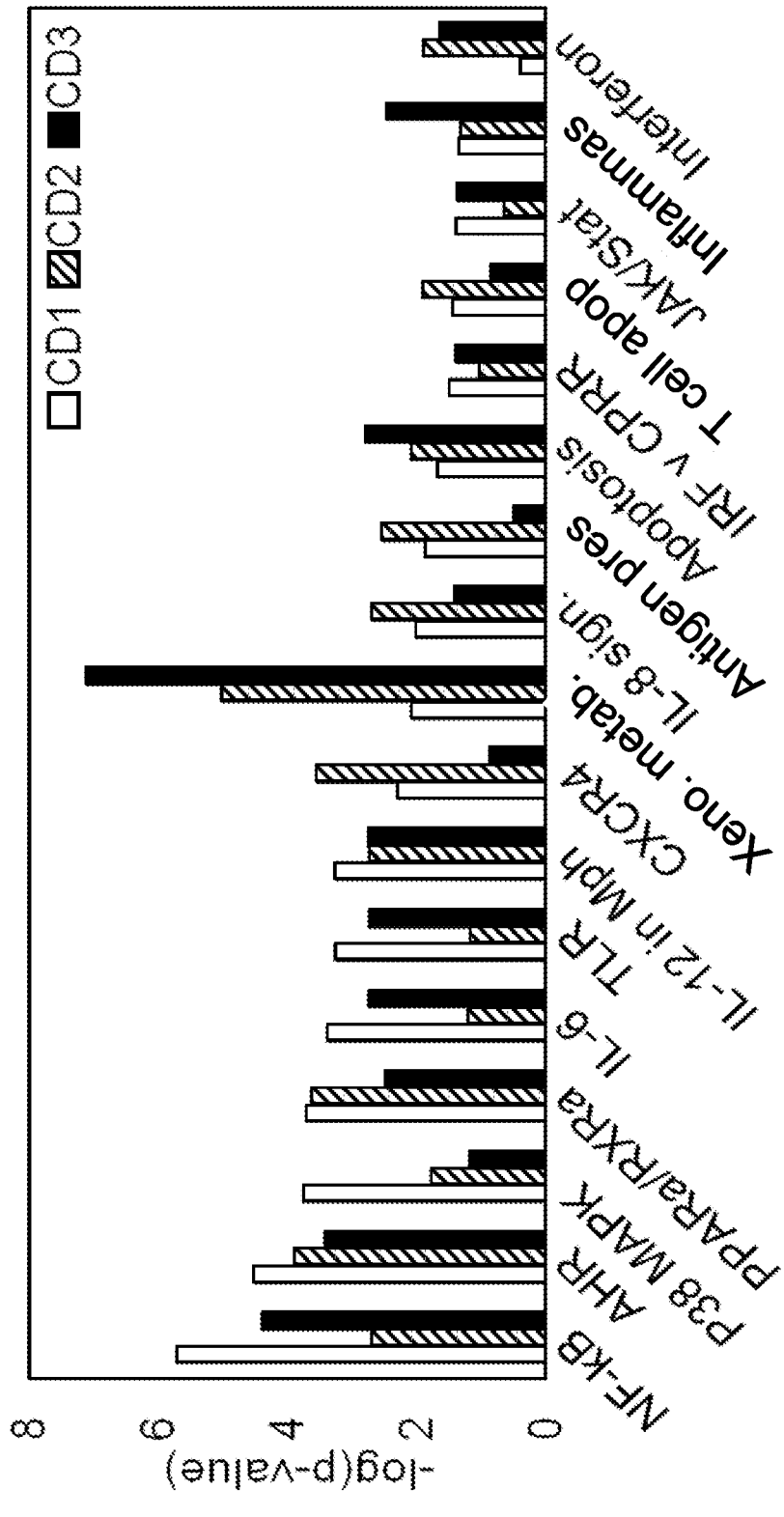
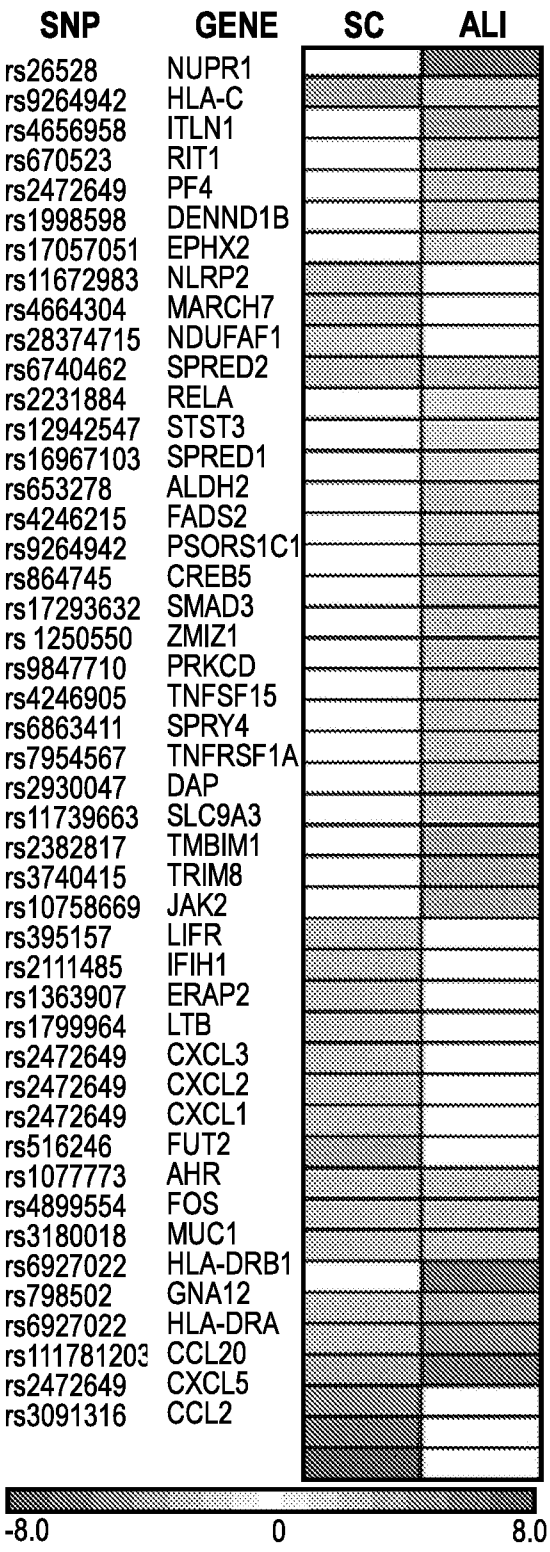

CD1
CD2
CD3
-log(p-value)
0
2
4
6
8
NF-kB
AHR
p38 MAPK
PPARa/RXRa
IL-6
TLR
IL-12 in Mph
CXCR4
Xeno. metab.
IL-8 sign.
Antigen pres
Apoptosis
IRF v CPRR
T cell apop
JAK/Stat
Inflammas
Interferon

Fig. 2D

Fig. 2E


SNP
GENE
SC
ALI
rs26528 NUPR1
rs9264942 HLA-C
rs4656958 ITLN1
rs670523 RIT1
rs2472649 PF4
rs1998598 DENND1B
rs17057051 EPHX2
rs11672983 NLRP2
rs4664304 MARCH7
rs28374715 NDUFAF1
rs6740462 SPRED2
rs2231884 RELA
rs12942547 STST3
rs16967103 SPRED1
rs653278 ALDH2
rs4246215 FADS2
rs9264942 PSORS1C1
rs864745 CREB5
rs17293632 SMAD3
rs 1250550 ZMIZ1
rs9847710 PRKCD
rs4246905 TNFSF15
rs6863411 SPRY4
rs7954567 TNFRSF1A
rs2930047 DAP
rs11739663 SLC9A3
rs2382817 TMBIM1
rs3740415 TRIM8
rs10758669 JAK2
rs395157 LIFR
rs2111485 IFIH1
rs1363907 ERAP2
rs1799964 LTB
rs2472649 CXCL3
rs2472649 CXCL2
rs2472649 CXCL1
rs516246 FUT2
rs1077773 AHR
rs4899554 FOS
rs3180018 MUC1
rs6927022 HLA-DRB1
rs798502 GNA12
rs6927022 HLA-DRA
rs111781203 CCL20
rs2472649 CXCL5
rs3091316 CCL2
-8.0
0
8.0

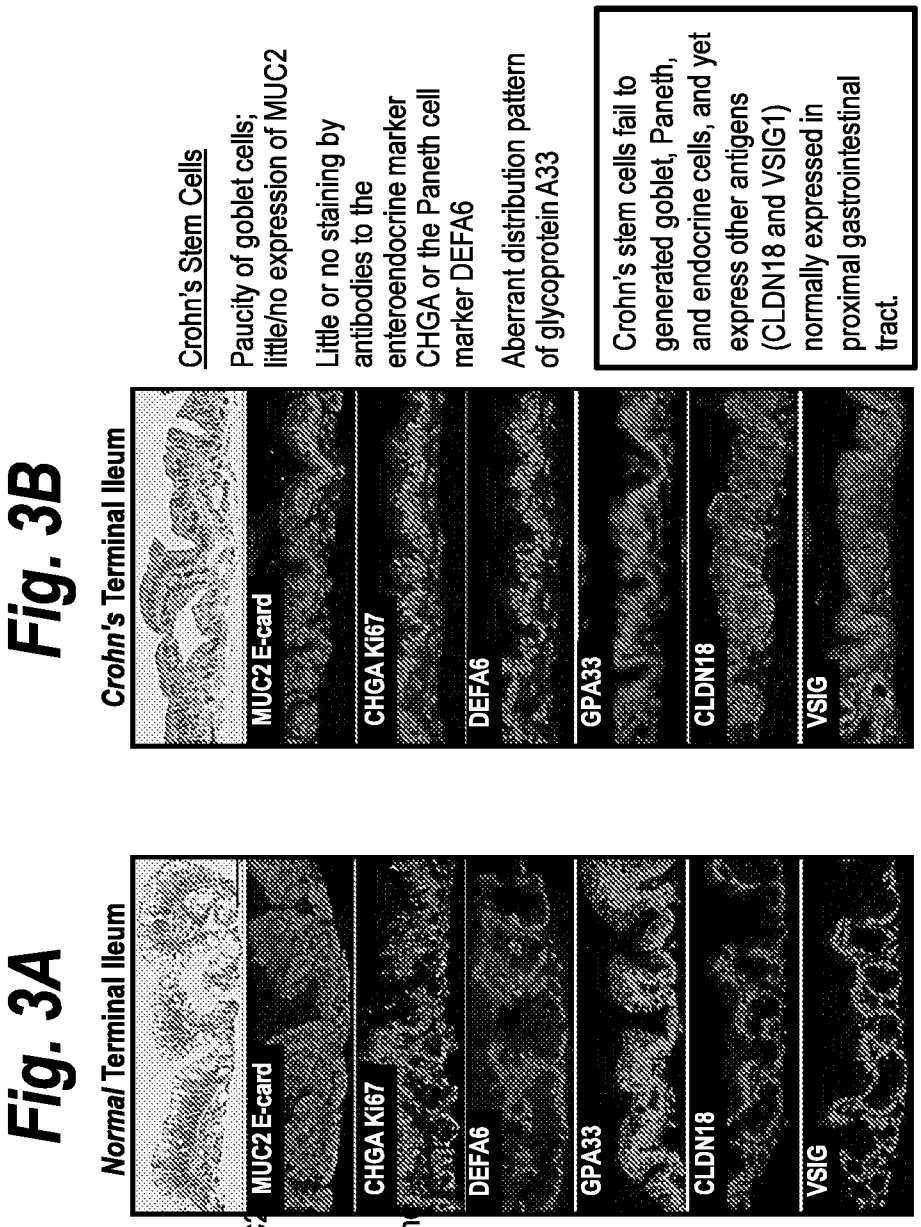


Fig. 3A
Normal Terminal Ileum
MUC2 E-card
CHGA Ki67
DEFA6
GPA33
CLDN18
VSIG
Normal Stem Cells
Mucosa dominated by MUC2 expressing goblet cells
Expression of markers for enteroendocrine cells (chromogranin A; CHGA) and Paneth cells (defensin 6A; DEF6A)
Fig. 3B
Crohn's Terminal Ileum
MUC2 E-card
CHGA Ki67
DEFA6
GPA33
CLDN18
VSIG
Crohn's Stem Cells
Paucity of goblet cells; little/no expression of MUC2
Little or no staining by antibodies to the enteroendocrine marker CHGA or the Paneth cell marker DEFA6
Aberrant distribution pattern of glycoprotein A33
Crohn's stem cells fail to generated goblet, Paneth, and endocrine cells, and yet express other antigens (CLDN18 and VSIG1) normally expressed in proximal gastrointestinal tract.

Fig. 3C

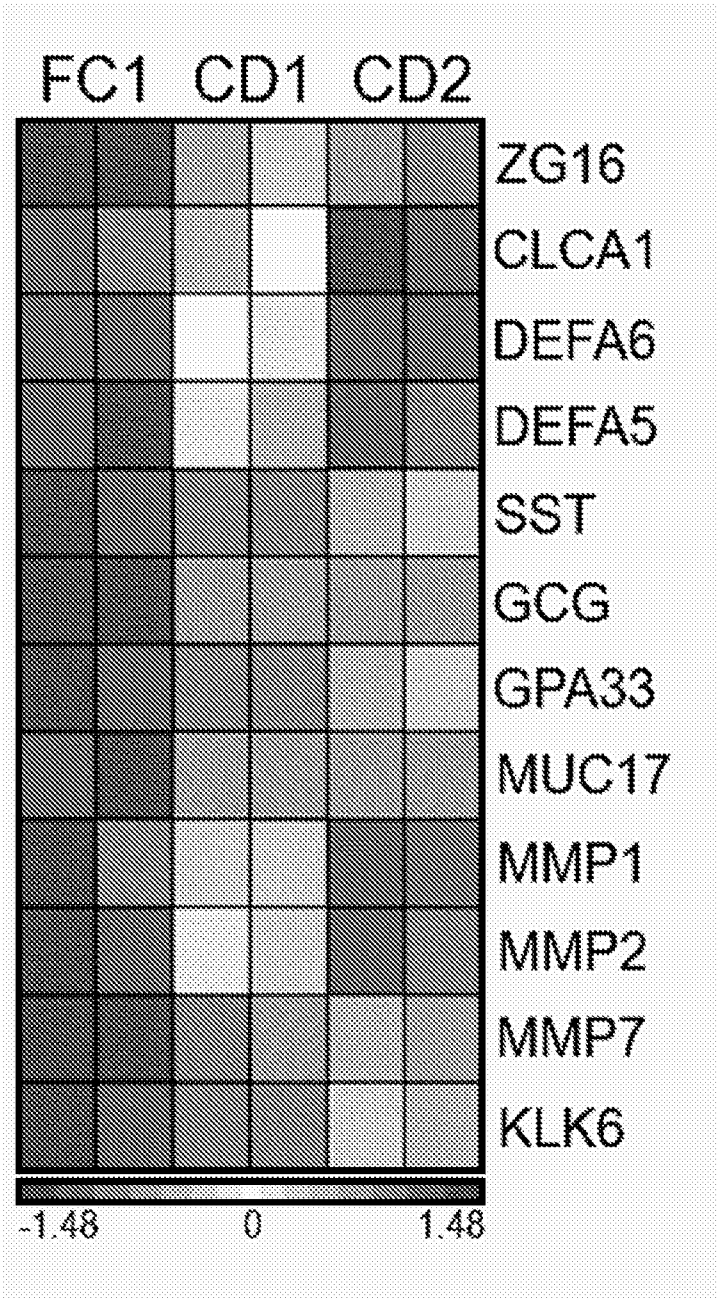

FC1
CD1
CD2
ZG16
CLCA1
DEFA6
DEFA5
SST
GCG
GPA33
MUC17
MMP1
MMP2
MMP7
KLK6
-1.48
0
1.48

Fig. 3D

Fig. 3E

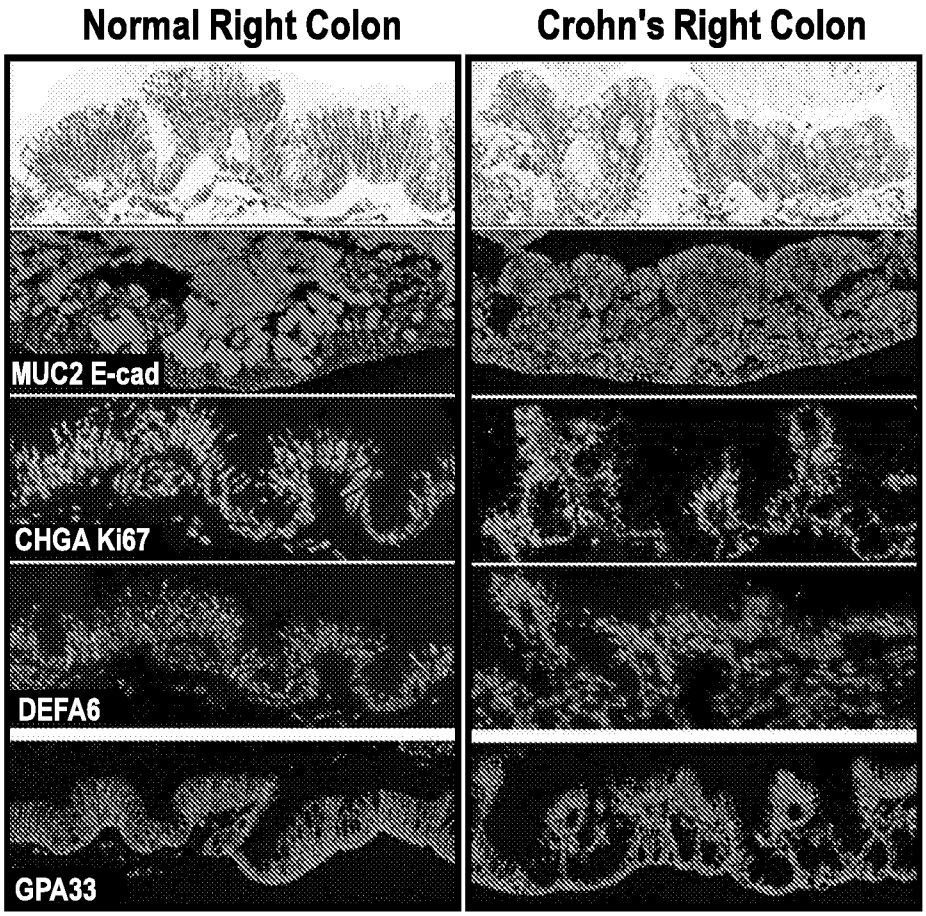

Normal Right Colon
Crohn's Right Colon
MUC2 E-cad
CHGA Ki67
DEFA6
GPA33

Fig. 3F

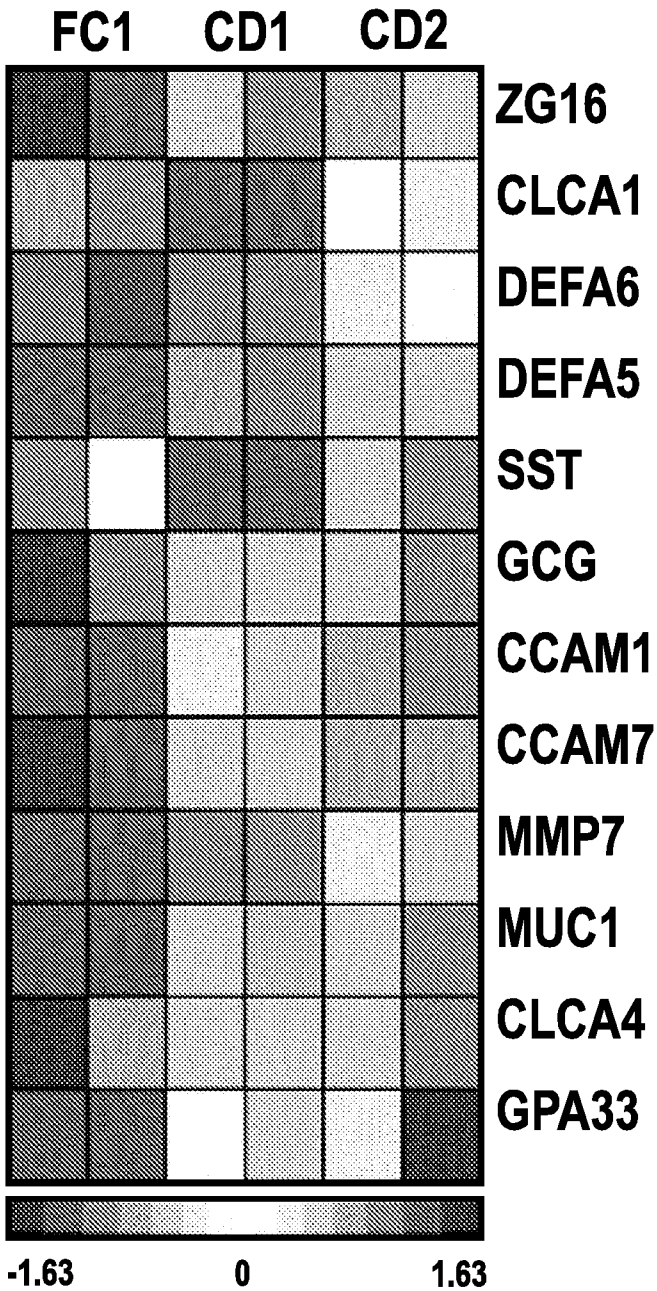
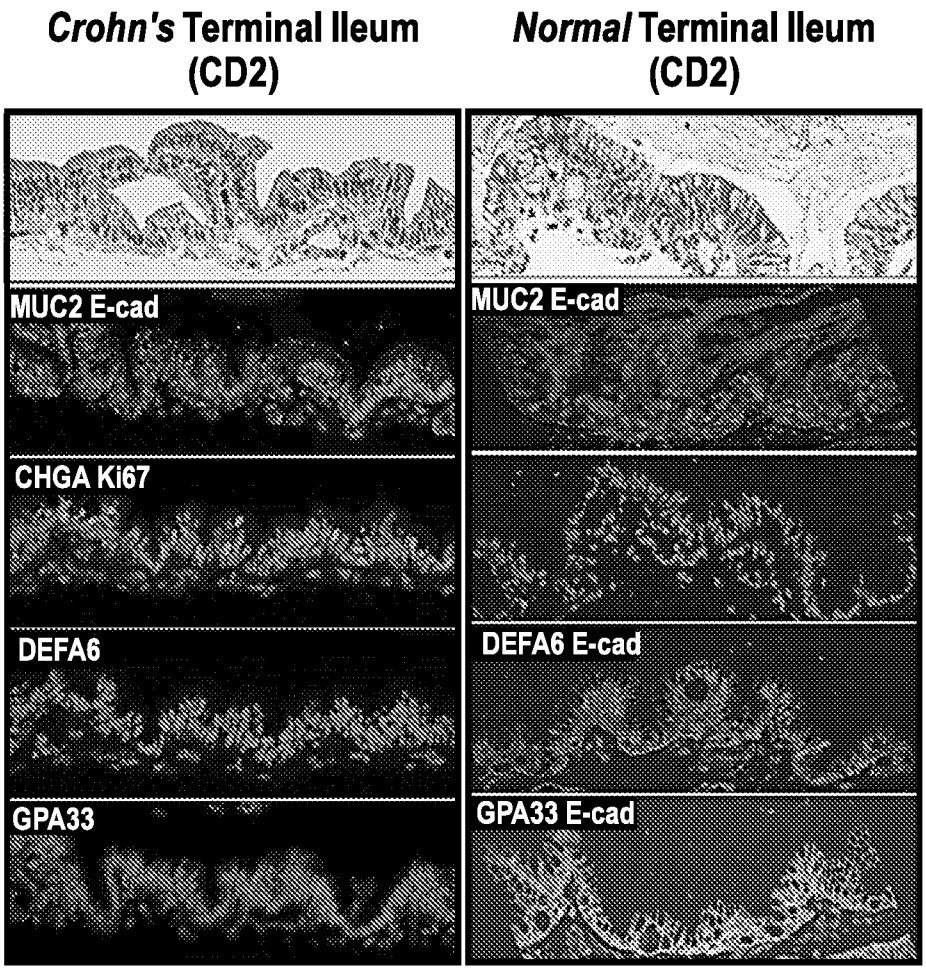

FC1
CD1
CD2
ZG16
CLCA1
DEFA6
DEFA5
SST
GCG
CCAM1
CCAM7
MMP7
MUC1
CLCA4
GPA33
-1.63
0
1.63

Fig. 3G


Crohn's Terminal Ileum (CD2)
Normal Terminal Ileum (CD2)
MUC2 E-cad
CHGA Ki67
DEFA6
GPA33
MUC2 E-cad
DEFA6 E-cad
GPA33 E-cad

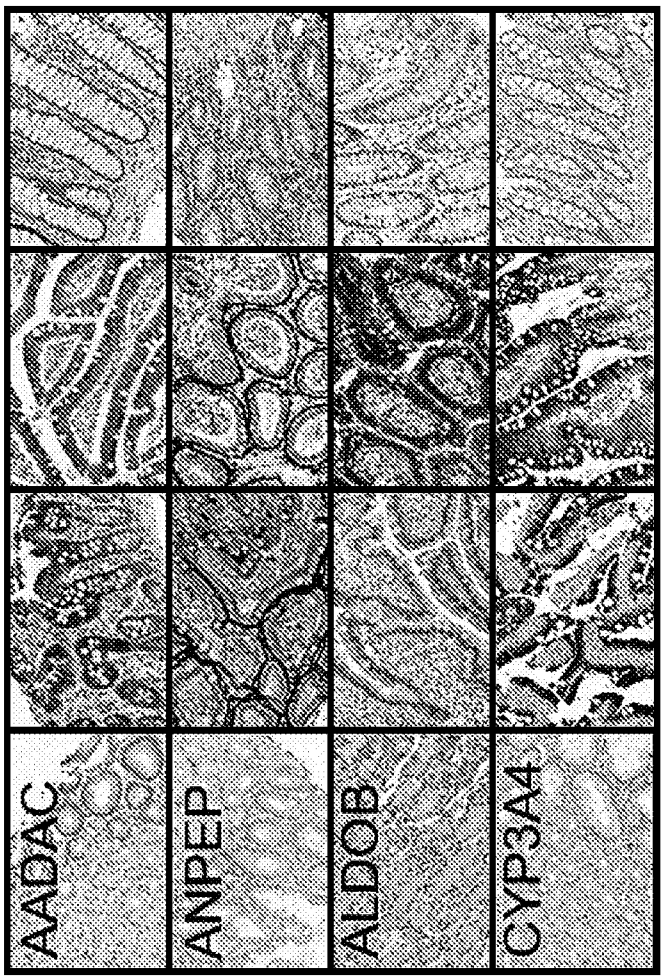
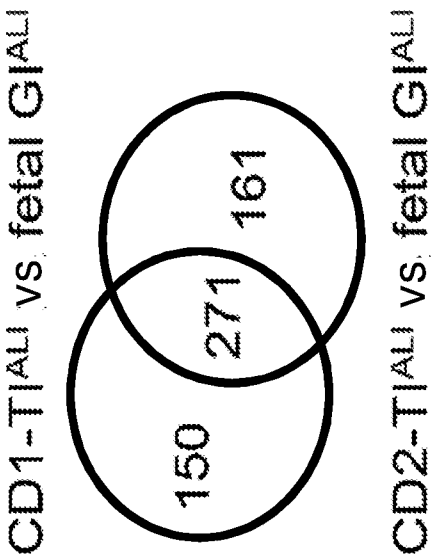


Fig. 4A

Fig. 4B



	p*z
Phase 1 comp. funct.	15.6
Metabolism	11.3
Biological oxidations	10.3
Lipid metabolism	6.3
Xenobiotics	5.5

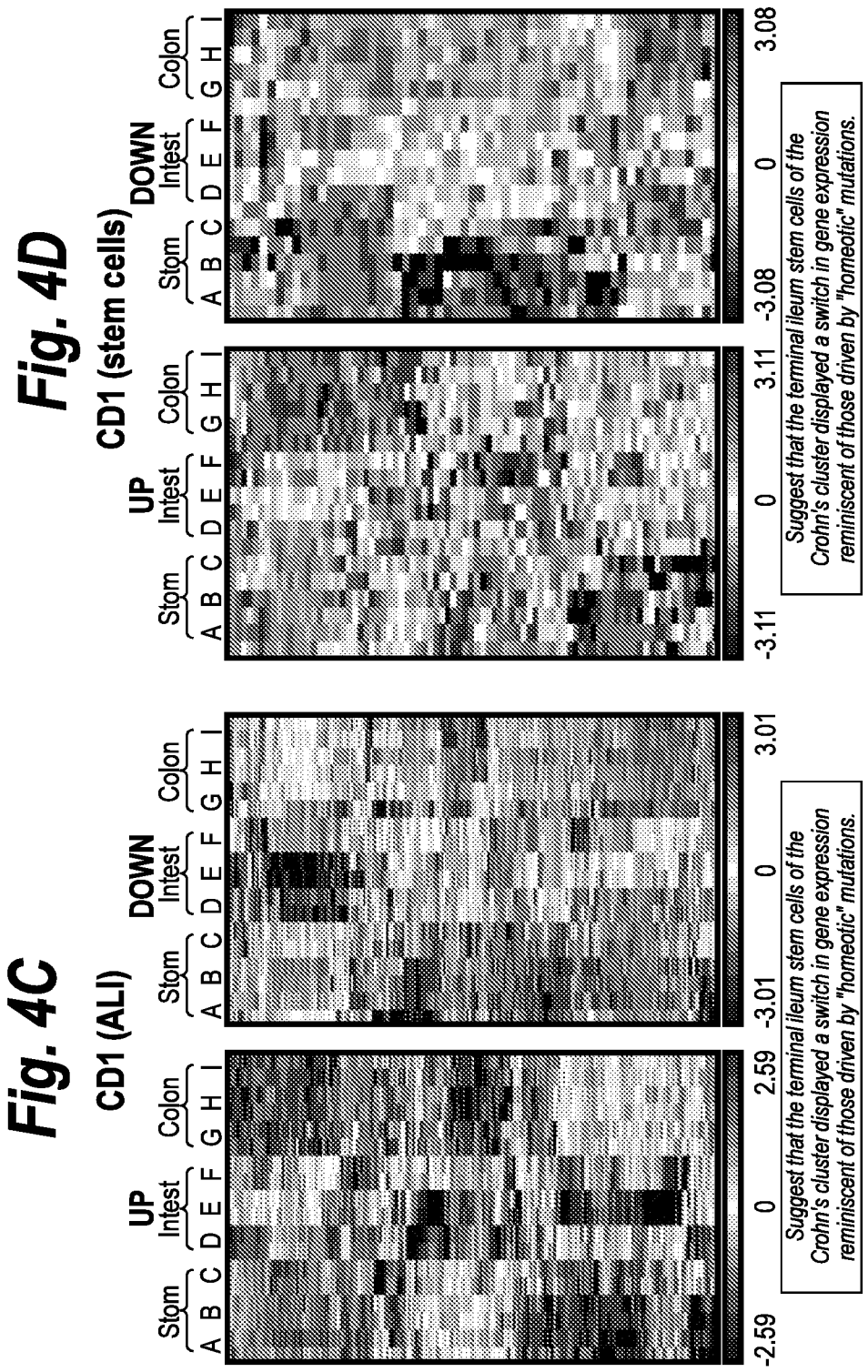


Fig. 4C
CD1 (ALI)
UP
DOWN
Stom
Intest
Colon
A B C D E F G H I
-2.59
0
2.59
-3.01
0
3.01
Suggest that the terminal ileum stem cells of the Crohn's cluster displayed a switch in gene expression reminiscent of those driven by "homeotic" mutations.
Fig. 4D
CD1 (stem cells)
UP
DOWN
Stom
Intest
Colon
A B C D E F G H I
-3.11
0
3.11
-3.08
0
3.08
Suggest that the terminal ileum stem cells of the Crohn's cluster displayed a switch in gene expression reminiscent of those driven by "homeotic" mutations.

Fig. 4E

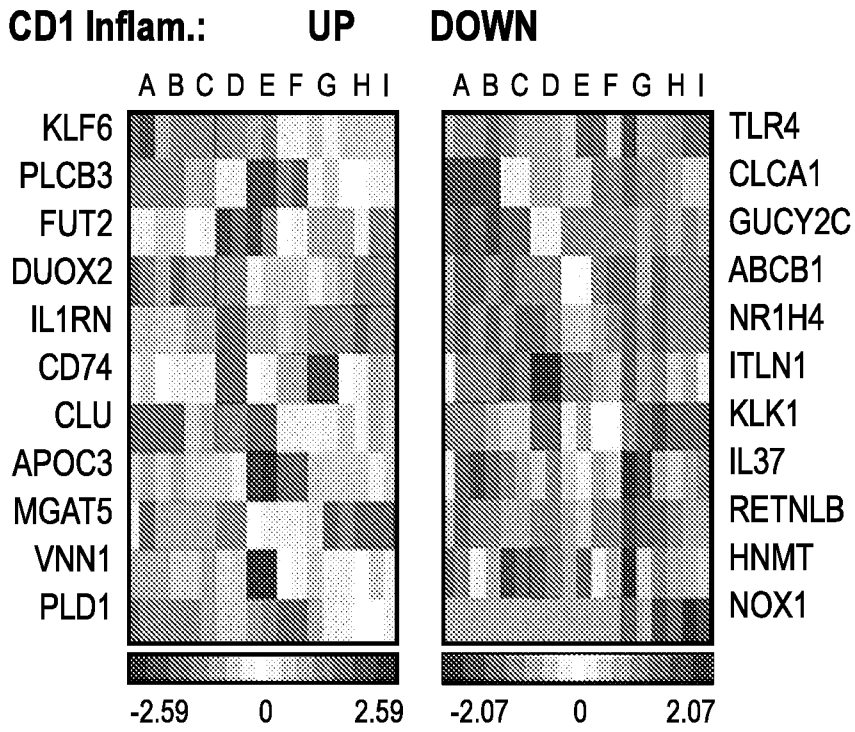

CD1 Inflam.:
UP
DOWN
A B C D E F G H I
A B C D E F G H I
KLF6
PLCB3
FUT2
DUOX2
IL1RN
CD74
CLU
APOC3
MGAT5
VNN1
PLD1
TLR4
CLCA1
GUCY2C
ABCB1
NR1H4
ITLN1
KLK1
IL37
RETNLB
HNMT
NOX1
-2.59
0
2.59
-2.07
0
2.07

Fig. 4F

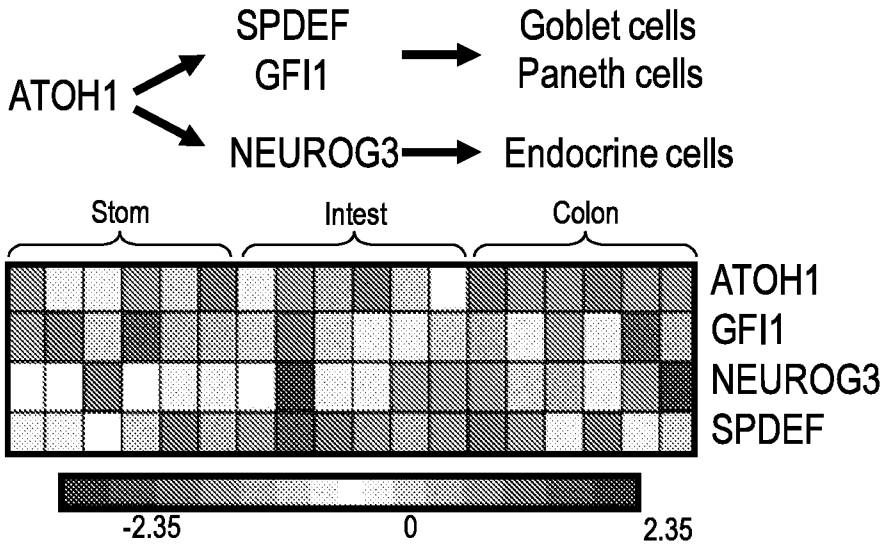

ATOH1
SPDEF
GFI1
Goblet cells
Paneth cells
NEUROG3
Endocrine cells
Stom
Intest
Colon
ATOH1
GFI1
NEUROG3
SPDEF
-2.35
0
2.35

Fig. 5A

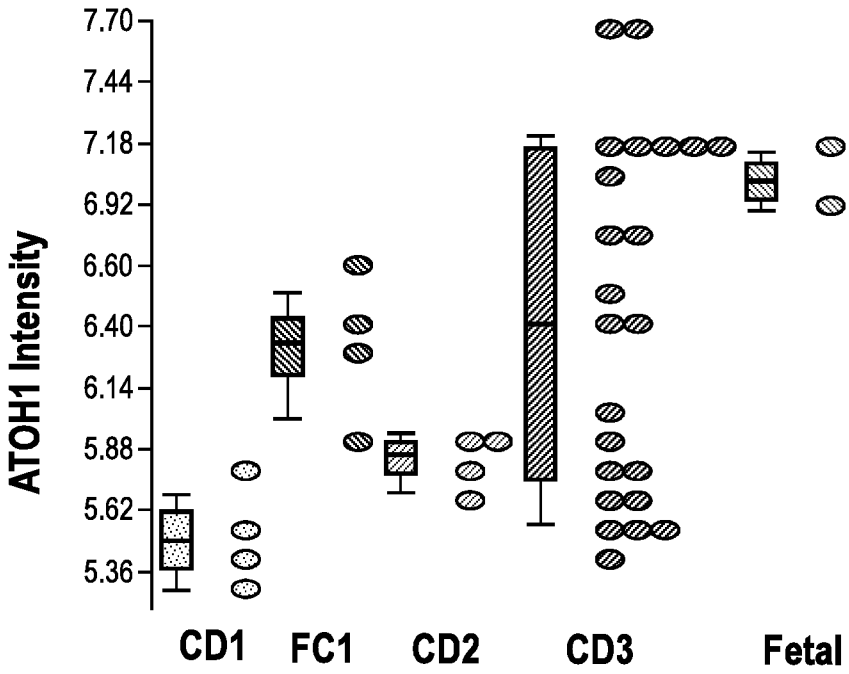

ATOH1 Intensity
7.70
7.44
7.18
6.92
6.60
6.40
6.14
5.88
5.62
5.36
CD1
FC1
CD2
CD3
Fetal

Fig. 5B

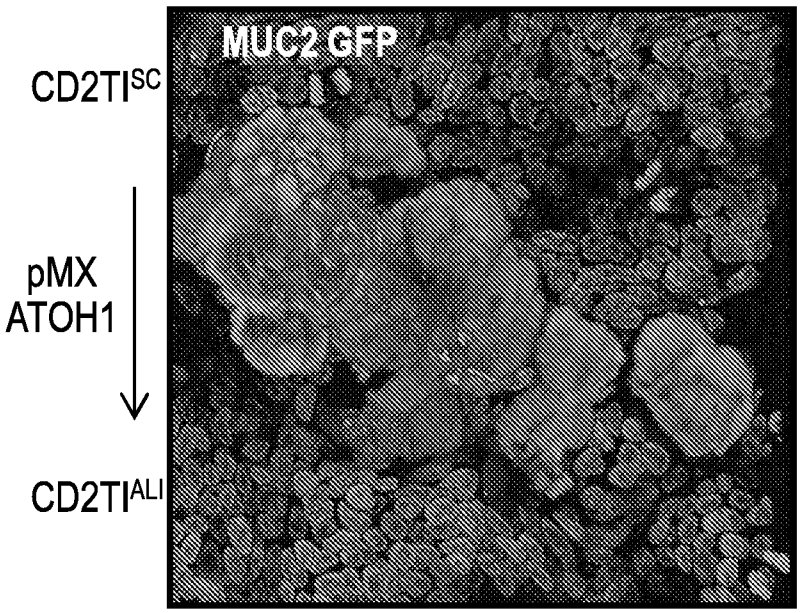

MUC2 GFP
CD2TI^SC
pMX
ATOH1
CD2TI^ALI

Fig. 5C

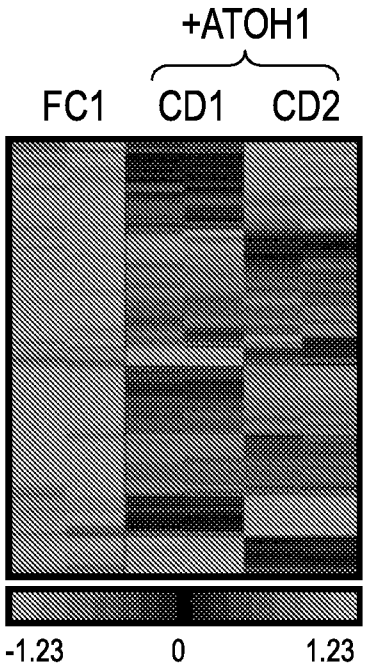


Fig. 5D

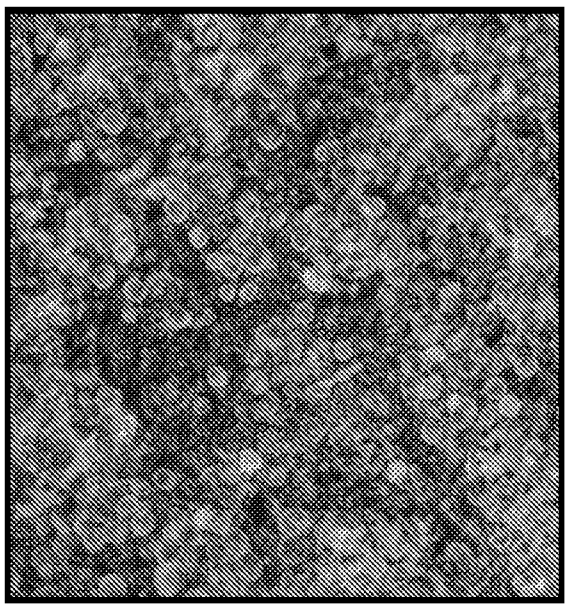


Fig. 5E

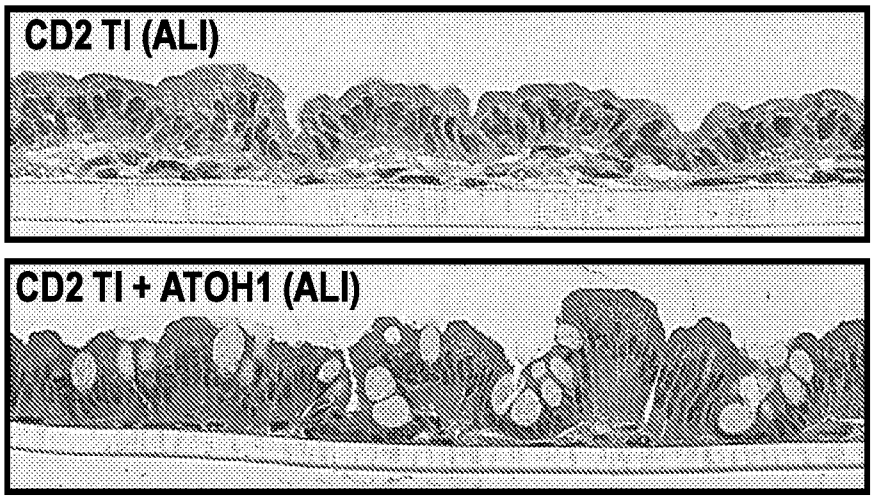
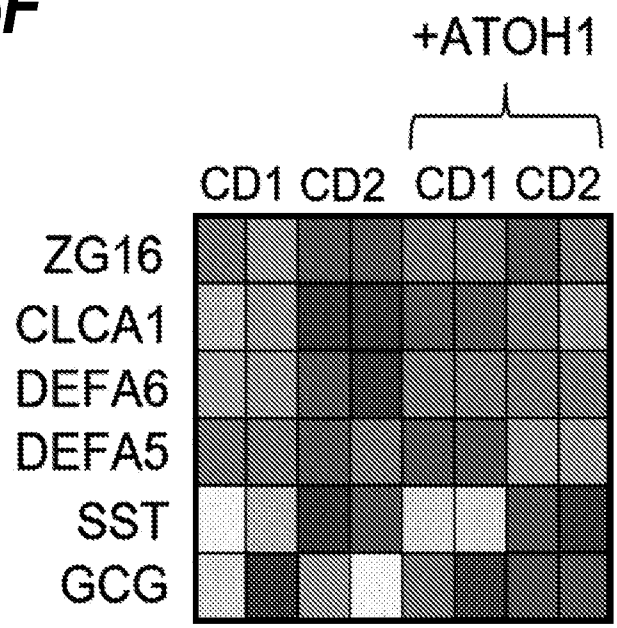

CD2 TI (ALI)
CD2 TI + ATOH1 (ALI)

Fig. 5F


+ATOH1
CD1 CD2 CD1 CD2
ZG16
CLCA1
DEFA6
DEFA5
SST
GCG

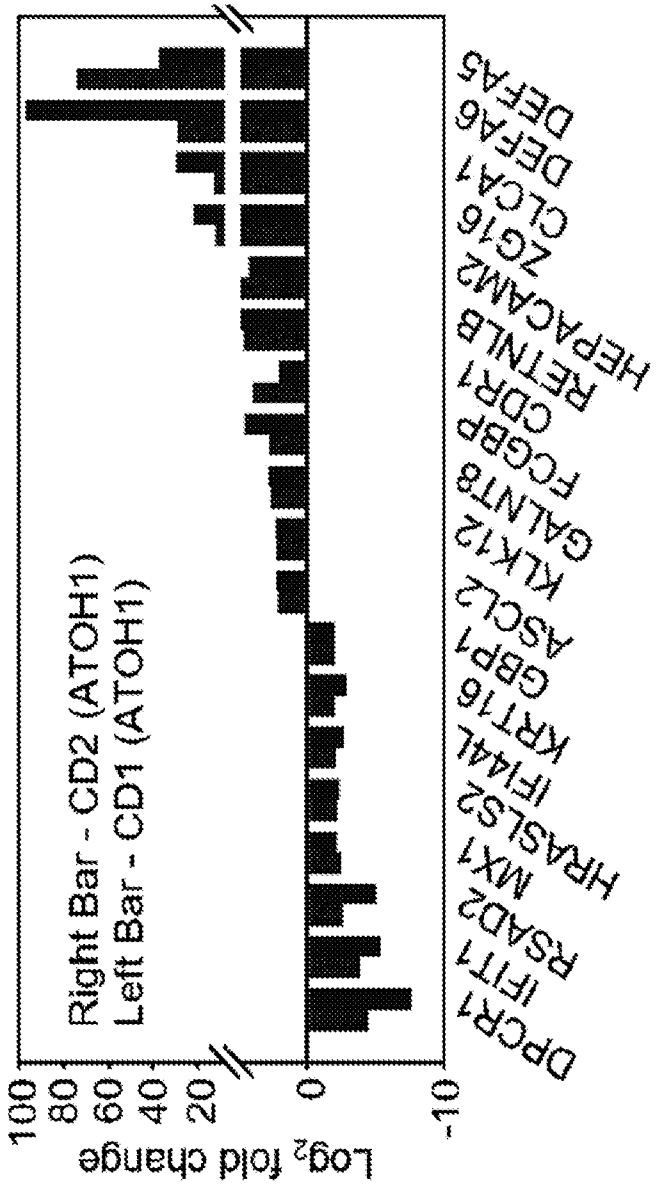

Log2 fold change
100
80
60
40
20
0
-10
Right Bar - CD2 (ATOH1)
Left Bar - CD1 (ATOH1)
DPCR1
IFIT1
RSAD2
MX1
HRASLS2
IFI44L
KRT16
GBP1
ASCL2
KLK12
GALNT8
FCGBP
CDR1
RETNLB
HEPACAM2
ZG16
CLCA1
DEFA6
DEFA5

Fig. 5G

Fig. 5H

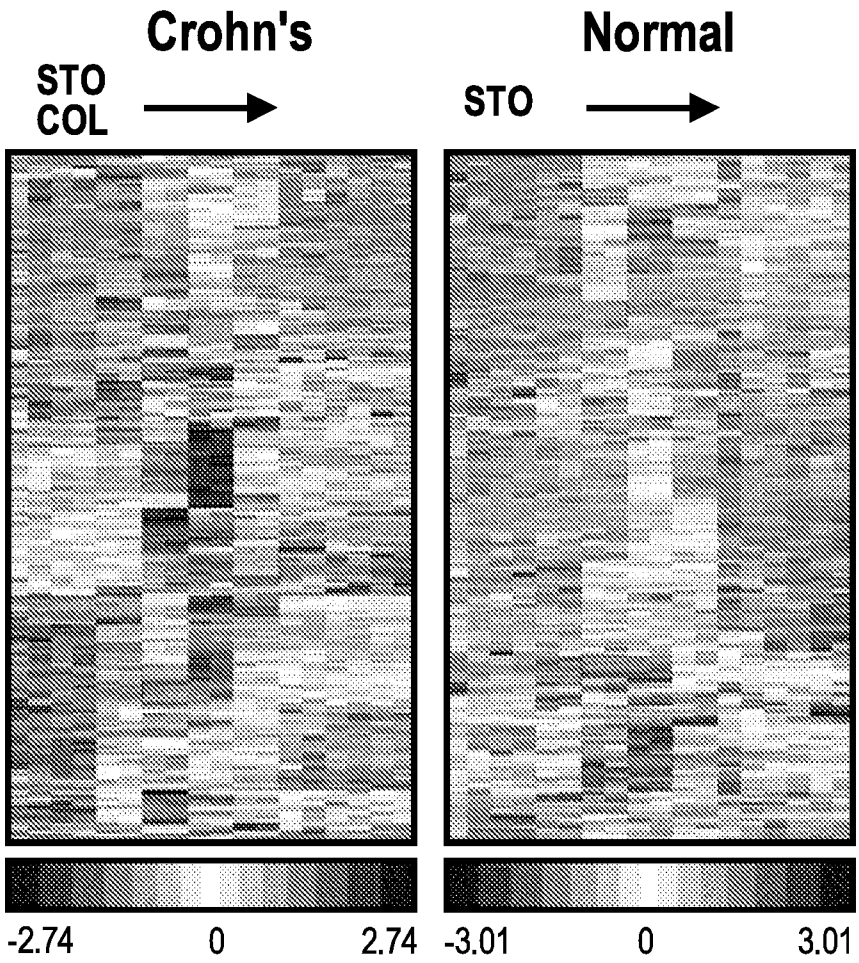
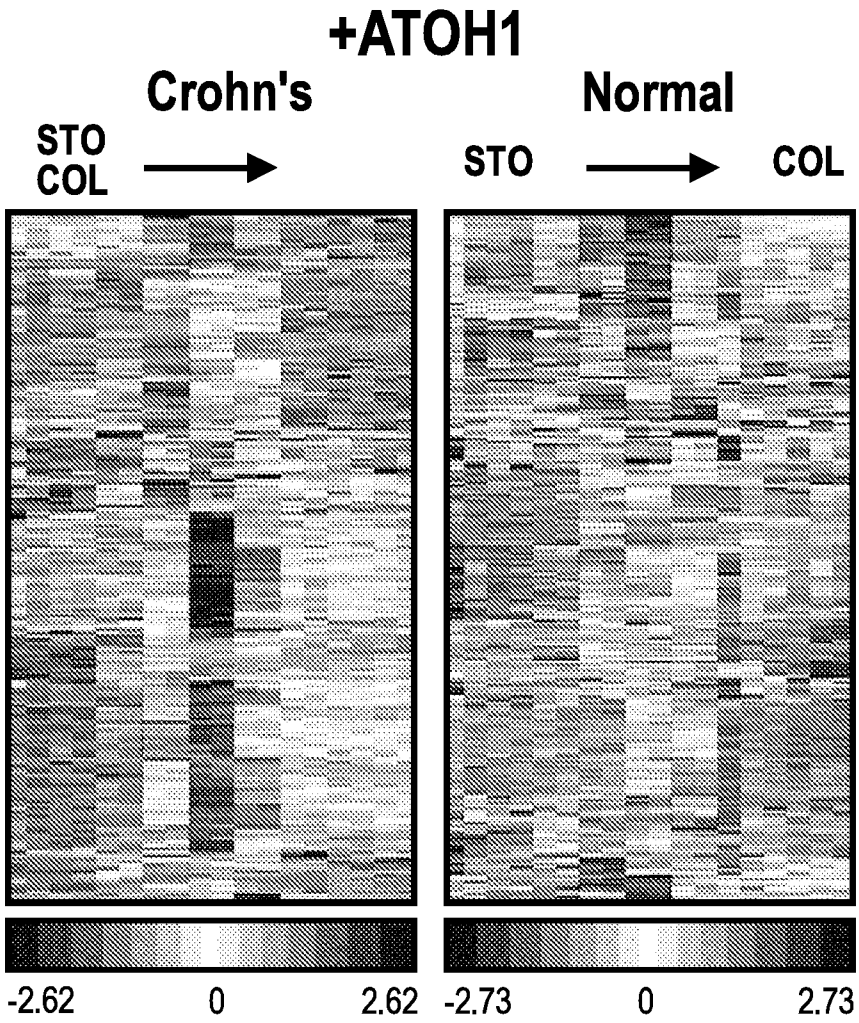


Fig. 5I



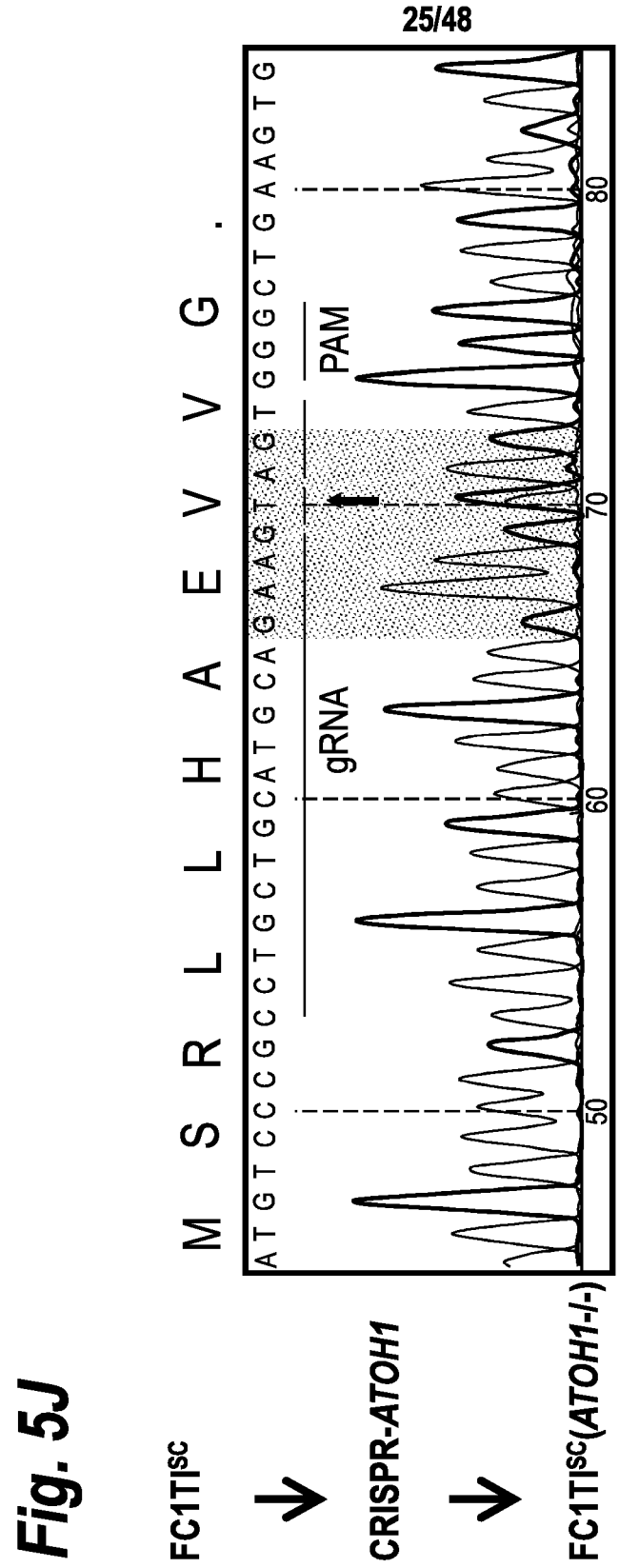

FC1TI^SC
CRISPR-ATOH1
FC1TI^SC(ATOH1-/-)
M S R L L H A E V V G .
A T G T C C C G C C T G C T G C A T G C A G A A G T A G T G G G C T G A A G T G
gRNA
PAM
50
60
70
80

Fig. 5J

Fig. 5K

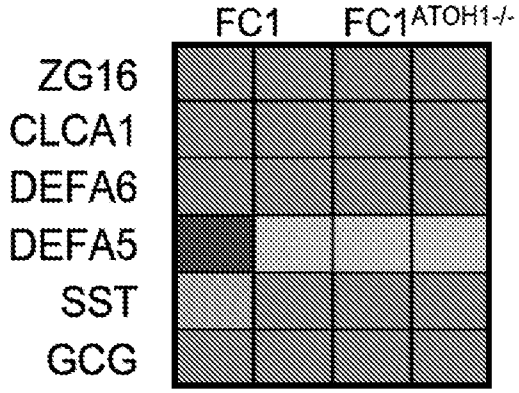
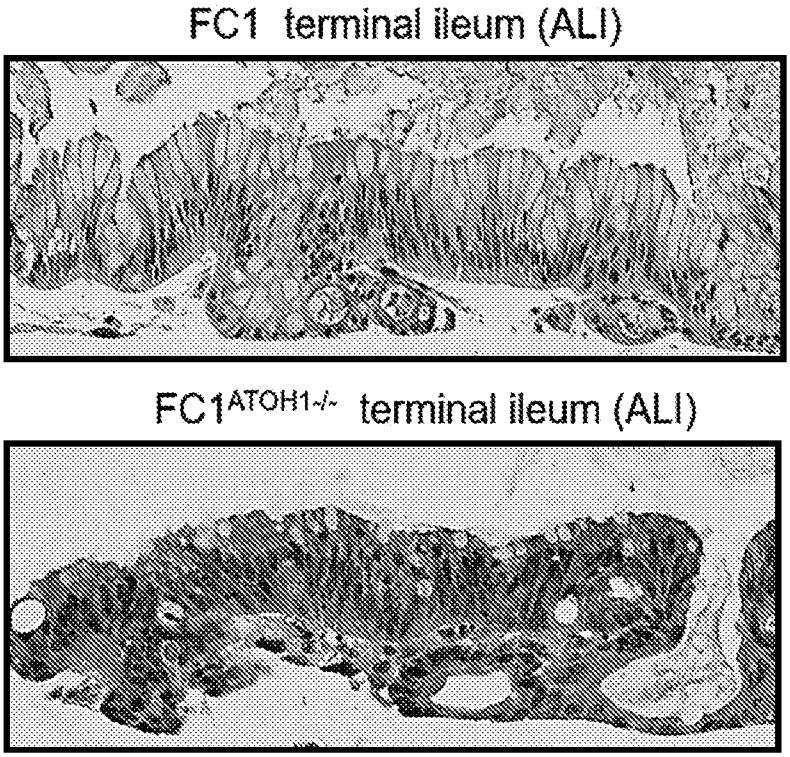

FC1
FC1ATOH1-/-
ZG16
CLCA1
DEFA6
DEFA5
SST
GCG

Fig. 5L


FC1 terminal ileum (ALI)
FC1ATOH1-/- terminal ileum (ALI)

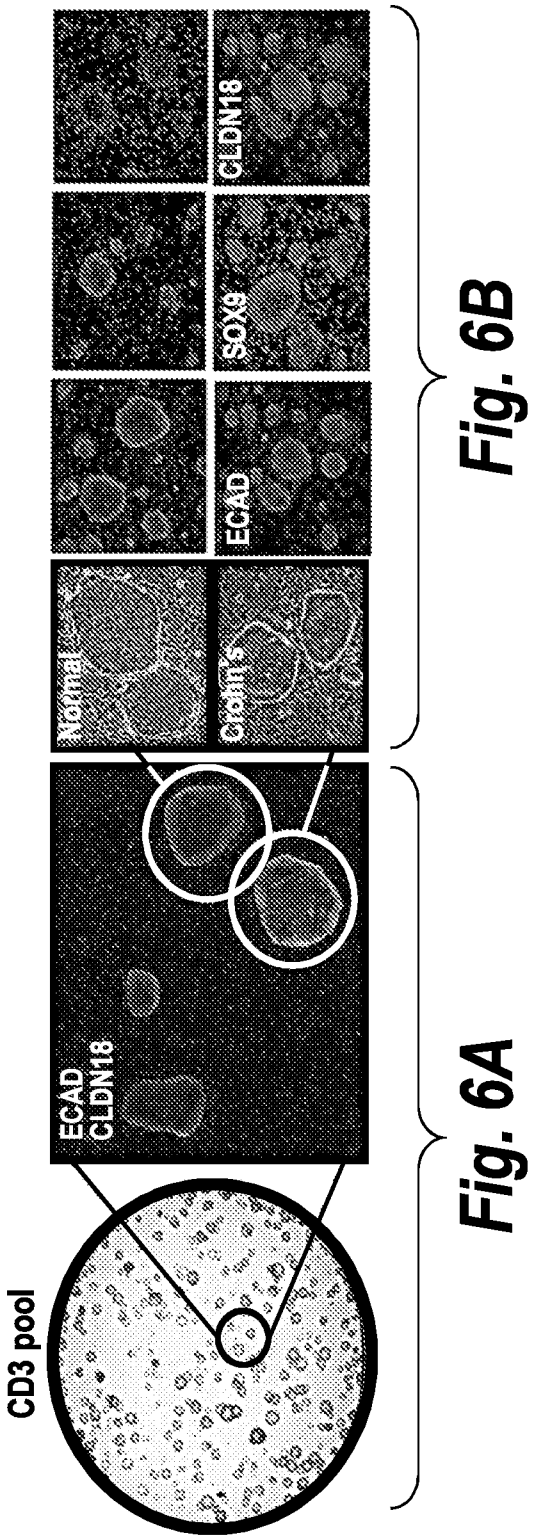


Fig. 6A

Fig. 6B

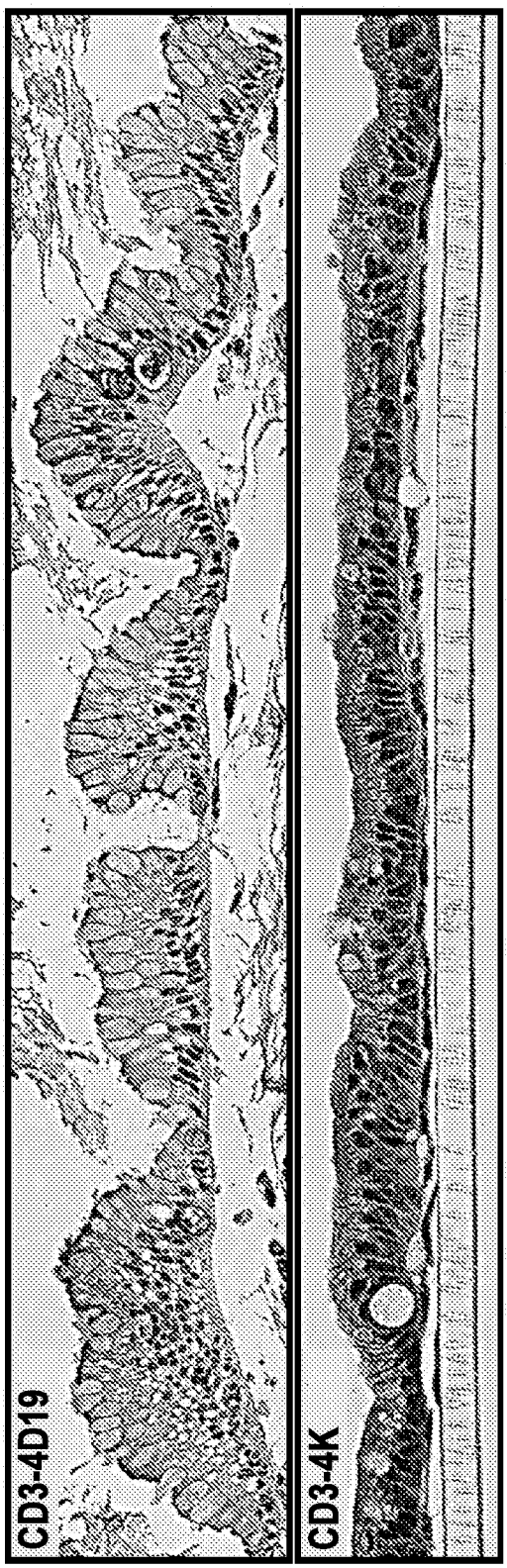


Fig. 6C

Fig. 6D

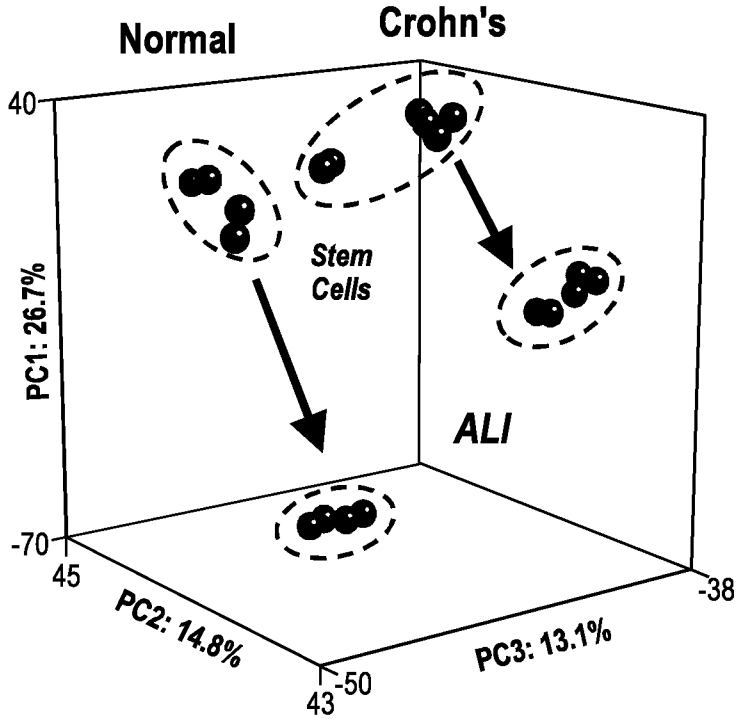

Normal
Crohn's
40
PC1: 26.7%
Stem
Cells
ALI
-70
45
PC2: 14.8%
43
-50
PC3: 13.1%
-38

Fig. 6E

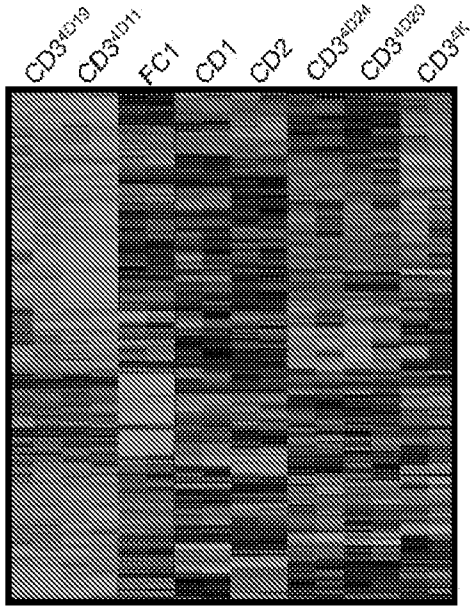

CD3 4D19
CD3 4D11
FC1
CD1
CD2
CD3 4D24
CD3 4D20
CD3 4K

Fig. 7

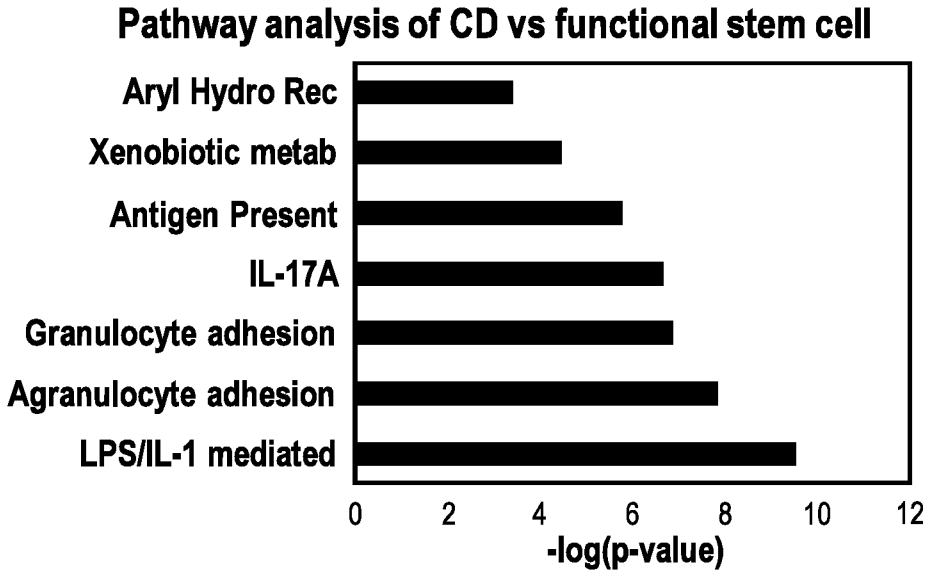

Pathway analysis of CD vs functional stem cell
Aryl Hydro Rec
Xenobiotic metab
Antigen Present
IL-17A
Granulocyte adhesion
Agranulocyte adhesion
LPS/IL-1 mediated
0
2
4
6
8
10
12
-log(p-value)

Fig. 8

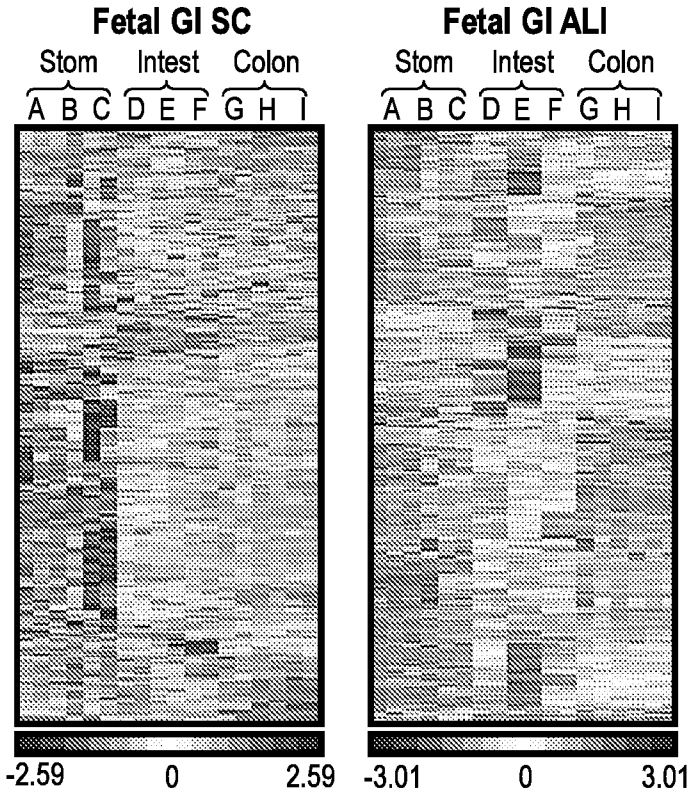

Fetal GI SC
Stom
Intest
Colon
A B C D E F G H I
-2.59
0
2.59
Fetal GI ALI
Stom
Intest
Colon
A B C D E F G H I
-3.01
0
3.01

Fig. 9

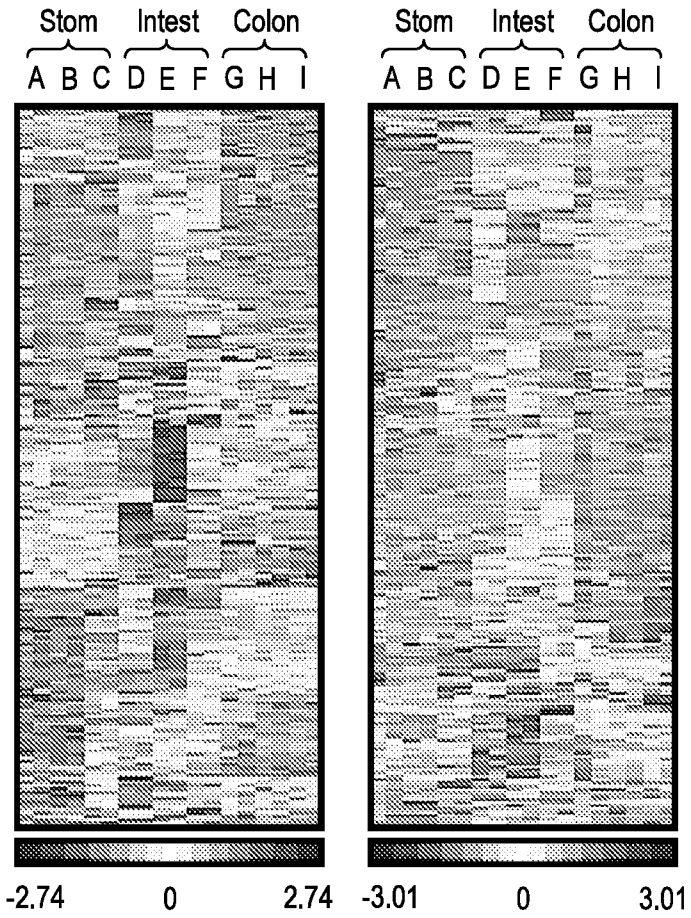

Stom
Intest
Colon
A B C D E F G H I
-2.74
0
2.74
Stom
Intest
Colon
A B C D E F G H I
-3.01
0
3.01

Fig. 10

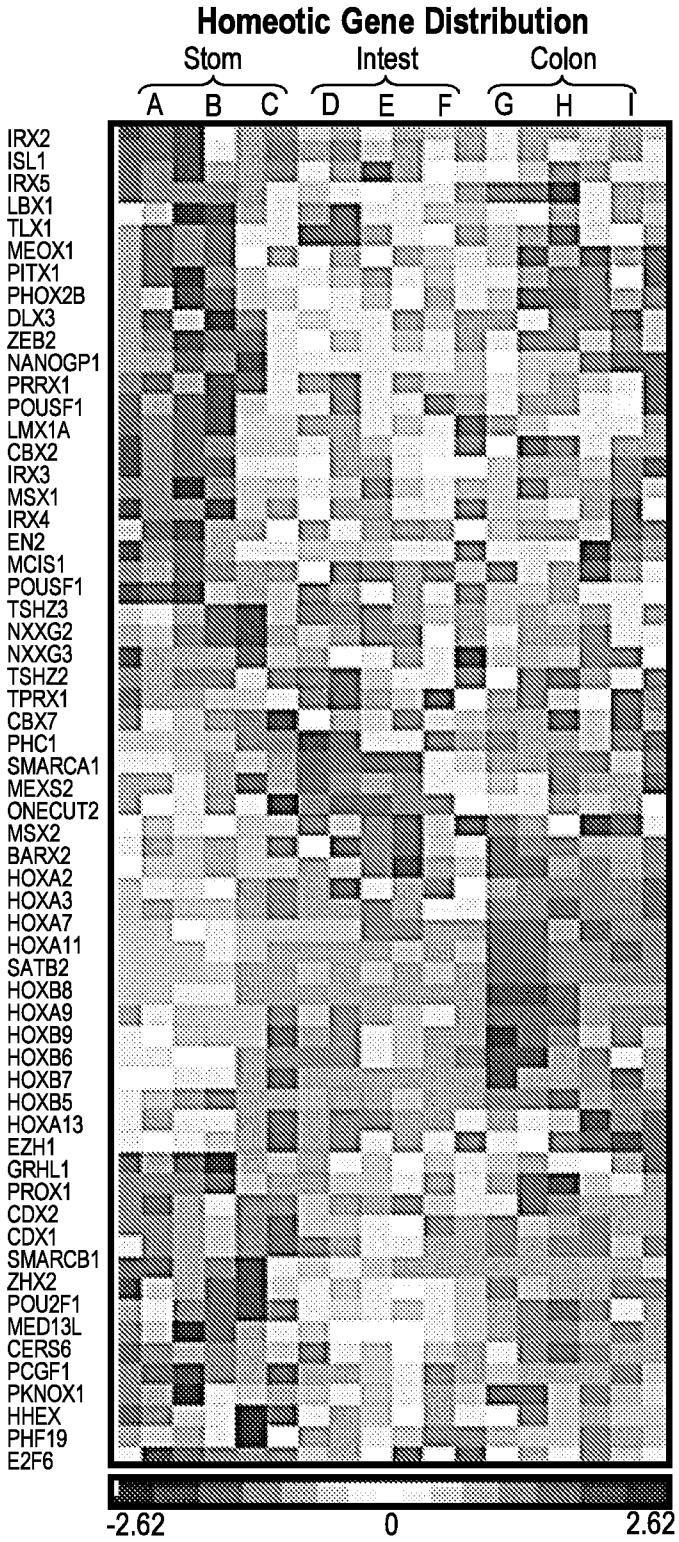
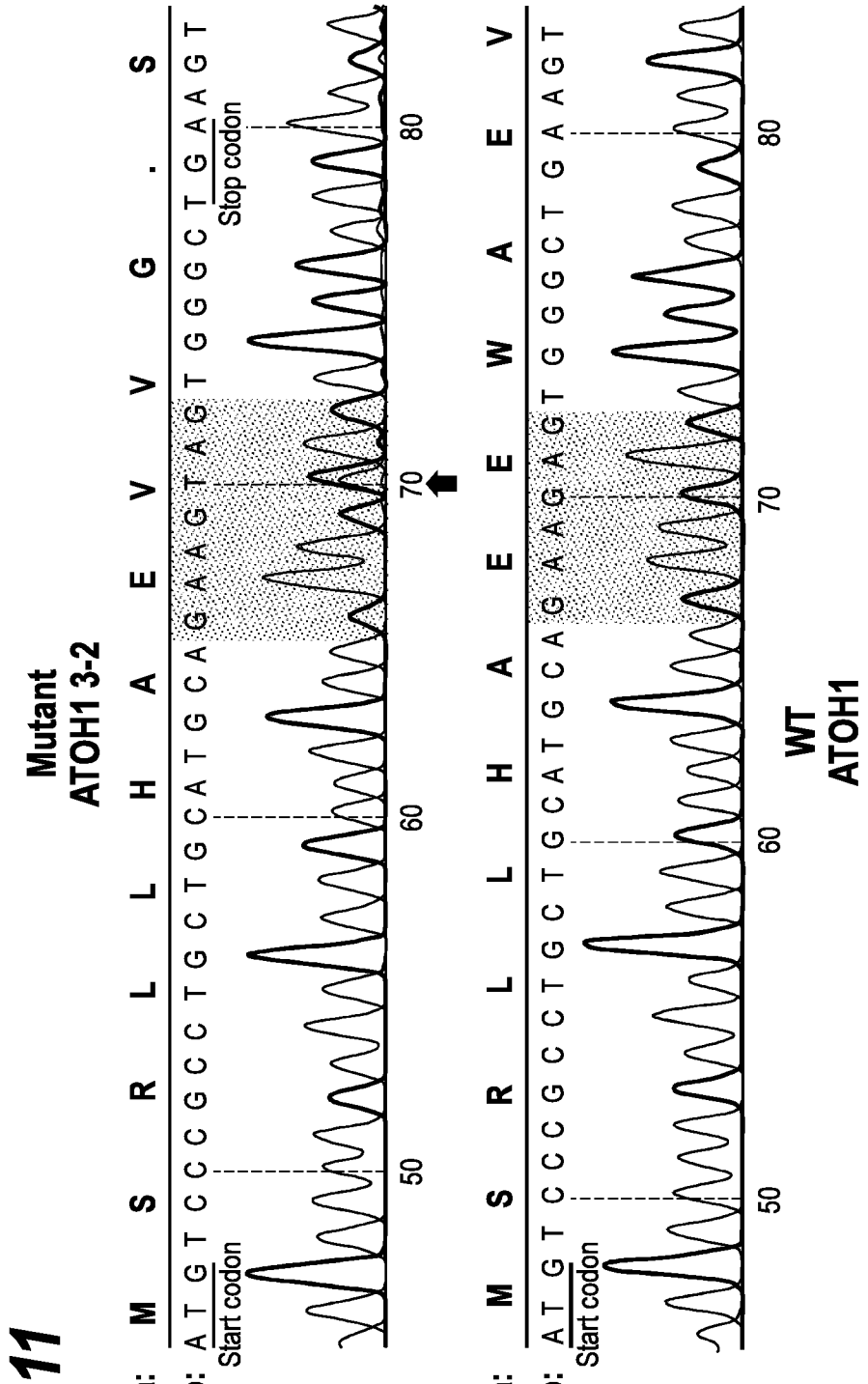

Homeotic Gene Distribution
Stom
Intest
Colon
A B C D E F G H I
IRX2
ISL1
IRX5
LBX1
TLX1
MEOX1
PITX1
PHOX2B
DLX3
ZEB2
NANOGP1
PRRX1
POUSF1
LMX1A
CBX2
IRX3
MSX1
IRX4
EN2
MCIS1
POUSF1
TSHZ3
NXXG2
NXXG3
TSHZ2
TPRX1
CBX7
PHC1
SMARCA1
MEXS2
ONECUT2
MSX2
BARX2
HOXA2
HOXA3
HOXA7
HOXA11
SATB2
HOXB8
HOXA9
HOXB9
HOXB6
HOXB7
HOXB5
HOXA13
EZH1
GRHL1
PROX1
CDX2
CDX1
SMARCB1
ZHX2
POU2F1
MED13L
CERS6
PCGF1
PKNOX1
HHEX
PHF19
E2F6
-2.62
0
2.62

Fig. 11


Mutant
ATOH1 3-2
aa: M S R L L H A E E V V G . S
bp: A T G T C C C G C C T G C T G C A T G C A G A A G T A G T G G G C T G A A G T
Start codon
Stop codon
50
60
70
80
WT
ATOH1
aa: M S R L L H A E E W A E V
bp: A T G T C C C G C C T G C T G C A T G C A G A A G A G T G G G C T G A A G T
Start codon
50
60
70
80

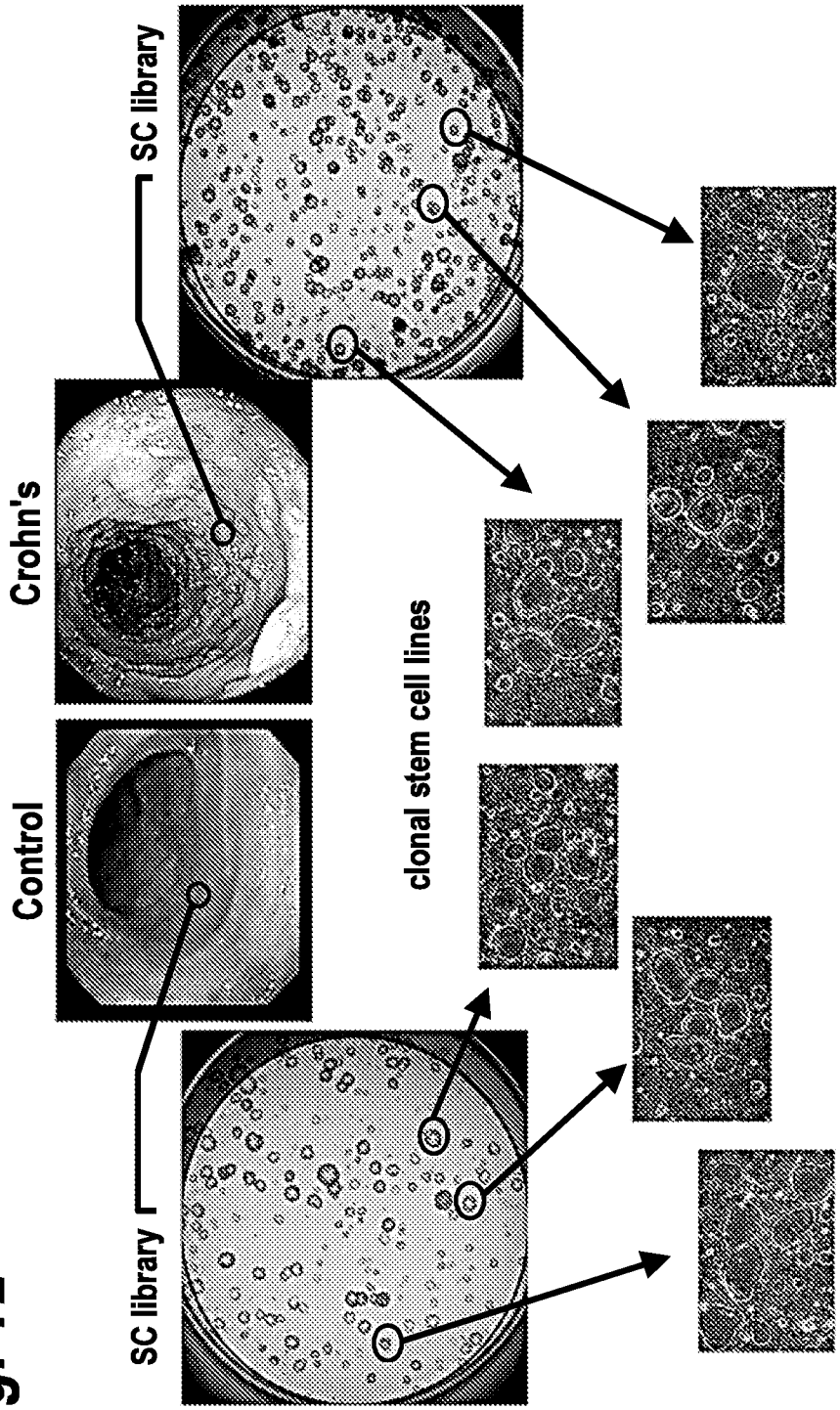

SC library
Crohn's
Control
clonal stem cell lines
SC library

Fig. 12

Fig. 13

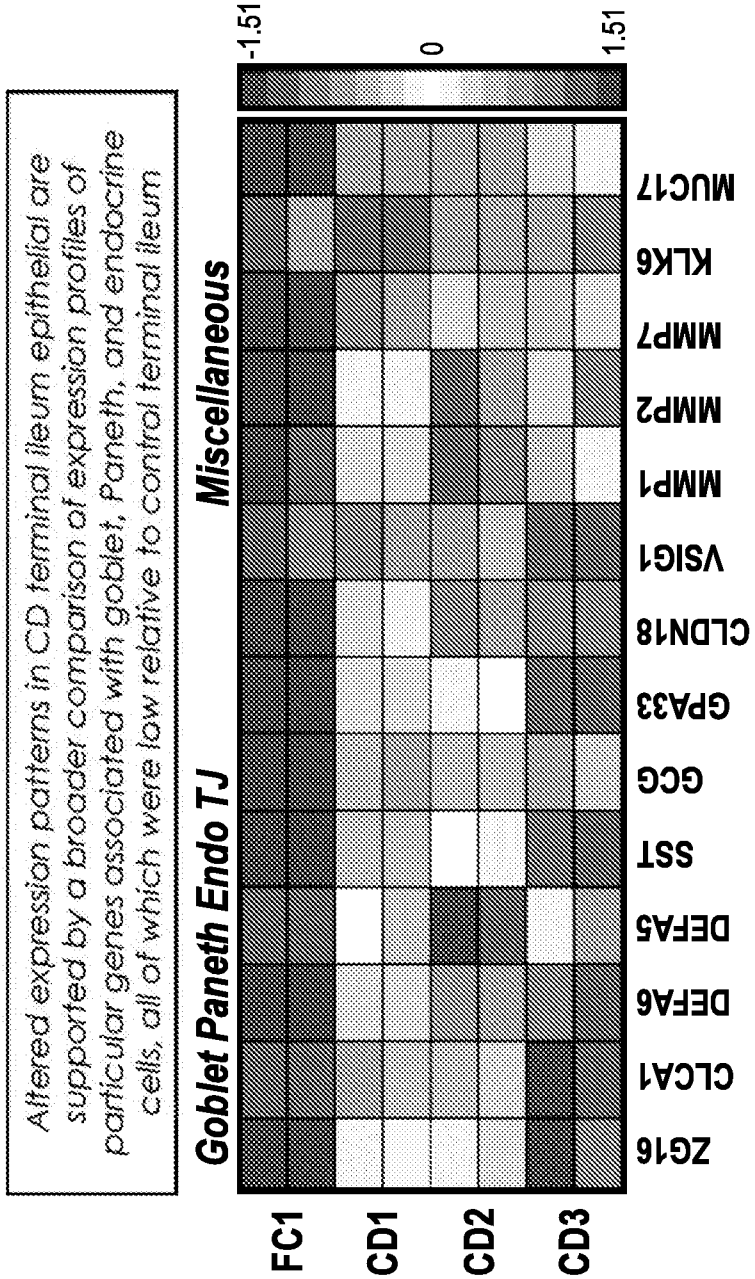

Altered expression patterns in CD terminal ileum epithelial are supported by a broader comparison of expression profiles of particular genes associated with goblet, Paneth, and endocrine cells, all of which were low relative to control terminal ileum
Goblet Paneth Endo TJ
Miscellaneous
-1.51
0
1.51
FC1
CD1
CD2
CD3
ZG16
CLCA1
DEFA6
DEFA5
SST
GCG
GPA33
CLDN18
VSIG1
MMP1
MMP2
MMP7
KLK6
MUC17

Fig. 14

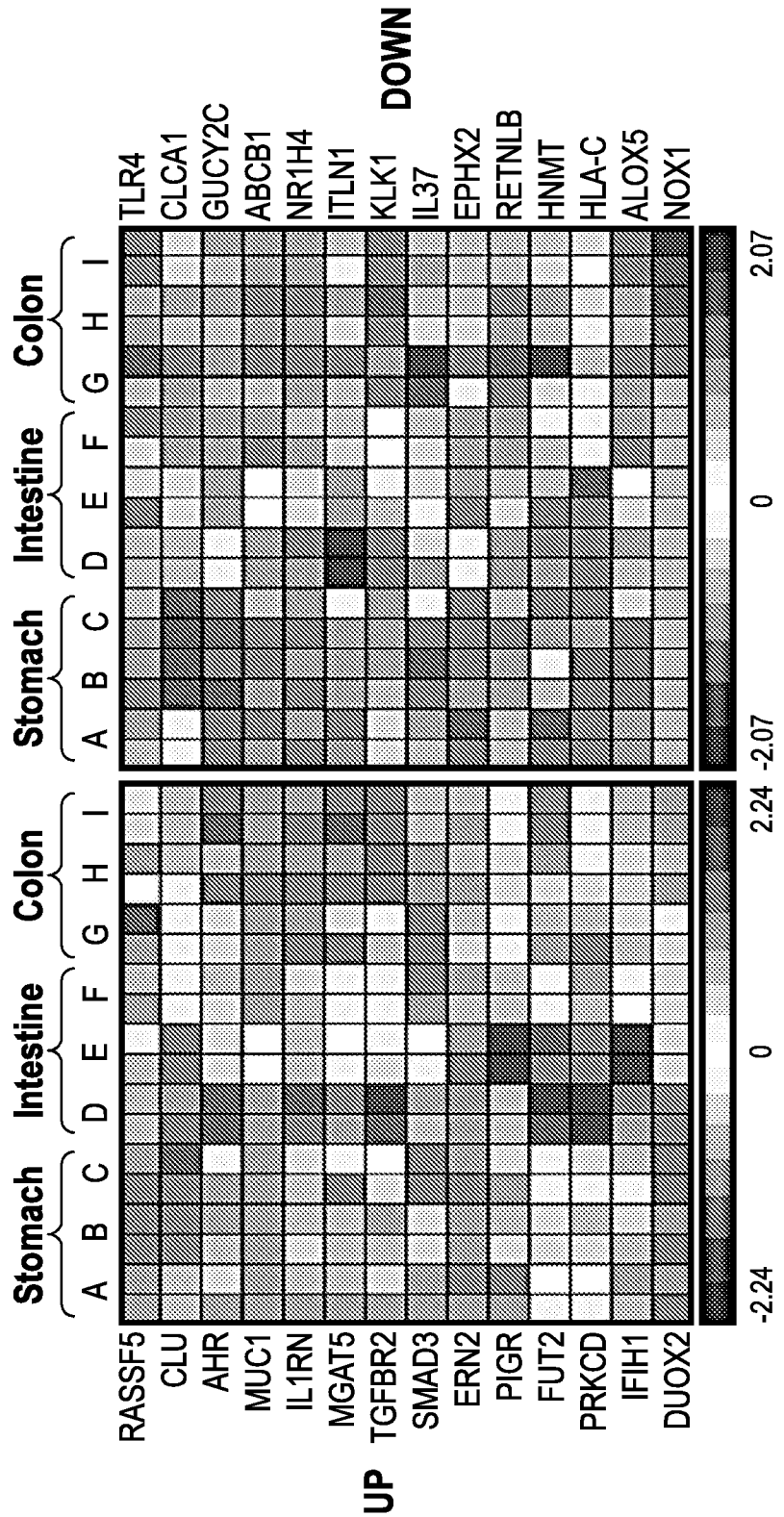

Stomach
Intestine
Colon
A B C D E F G H I
UP
RASSF5
CLU
AHR
MUC1
IL1RN
MGAT5
TGFBR2
SMAD3
ERN2
PIGR
FUT2
PRKCD
IFIH1
DUOX2
-2.24
0
2.24
DOWN
TLR4
CLCA1
GUCY2C
ABCB1
NR1H4
ITLN1
KLK1
IL37
EPHX2
RETNLB
HNMT
HLA-C
ALOX5
NOX1
-2.07
0
2.07

Fig. 15A

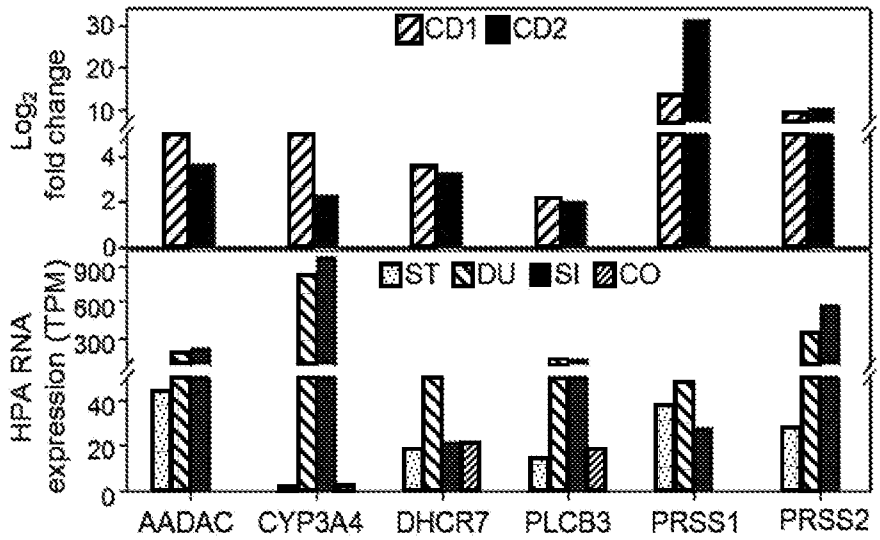


Fig. 15B

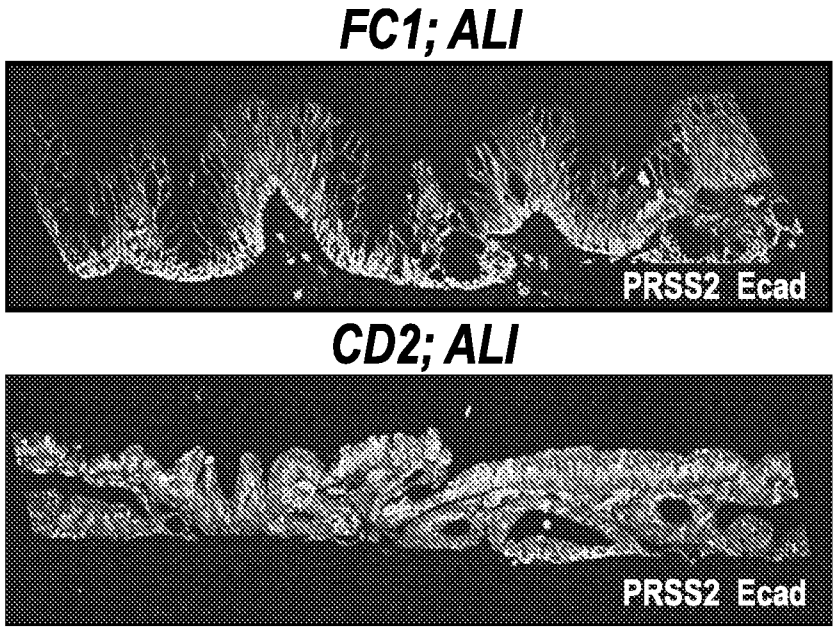


Fig. 16A

Using whole-genome analyses of epigenetic histone marks, we identified multiple alterations in the epigenetic profiles of the HOX loci of stem cells of the Crohn's cluster compared to those of the Normal cluster

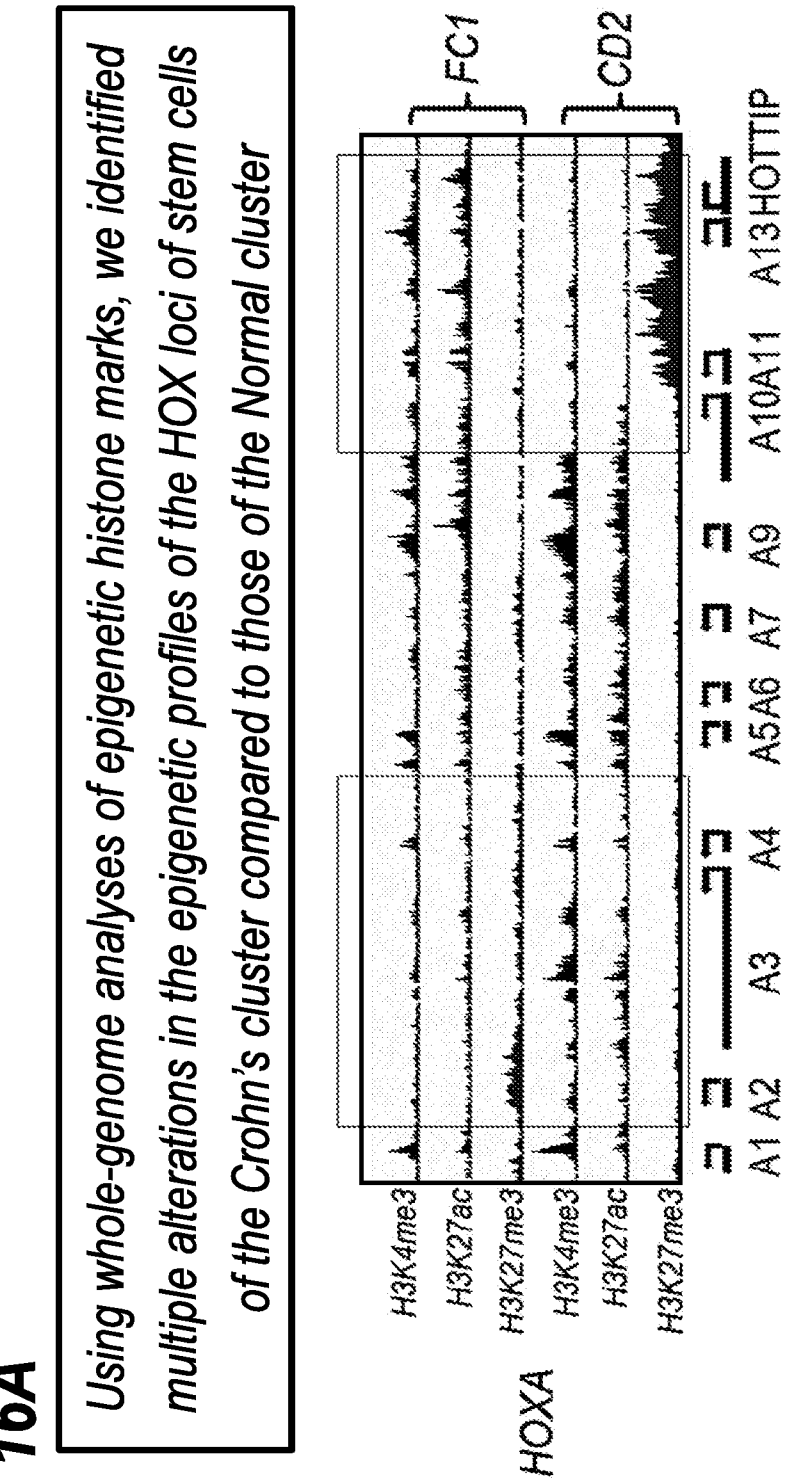


Fig. 16B

Among these genes are a host of transcription factors including CDX2 and GATA5, whose respective roles distal and proximal gastrointestinal tract differentiation are well established

Fig. 16C

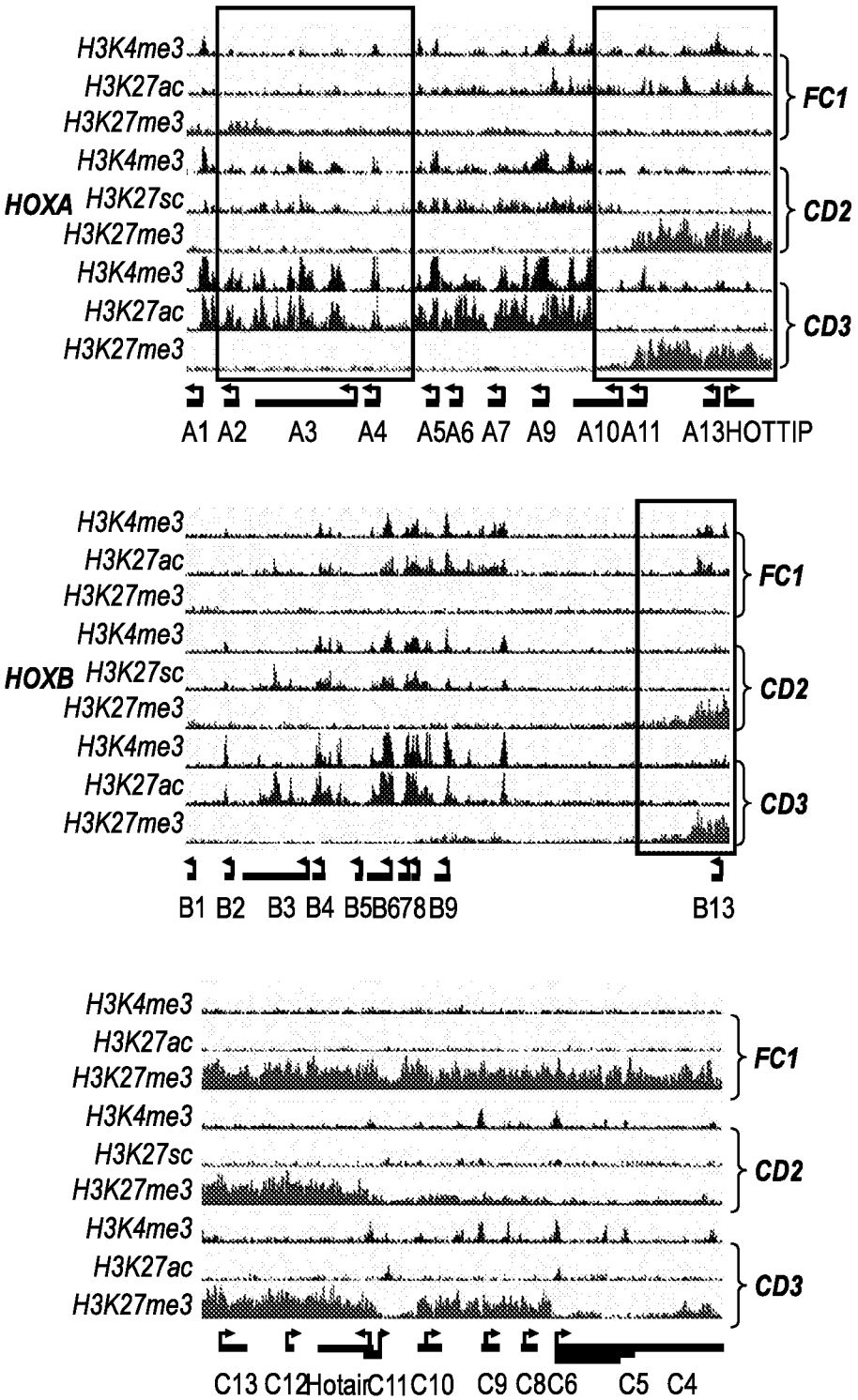

H3K4me3
H3K27ac
H3K27me3
FC1
HOXA
H3K27sc
CD2
CD3
A1 A2 A3 A4 A5A6 A7 A9 A10A11 A13HOTTIP
HOXB
B1 B2 B3 B4 B5B678B9 B13
C13 C12HotairC11C10 C9 C8C6 C5 C4

Fig. 17A

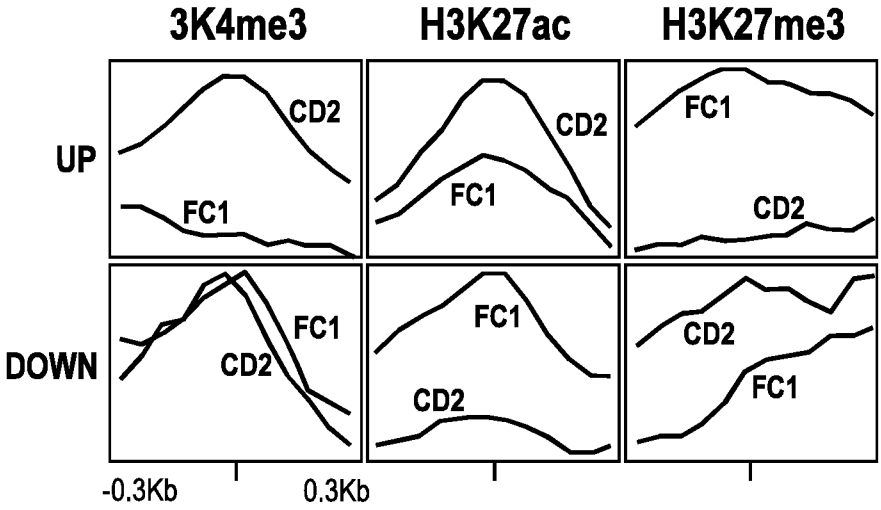


Fig. 17B

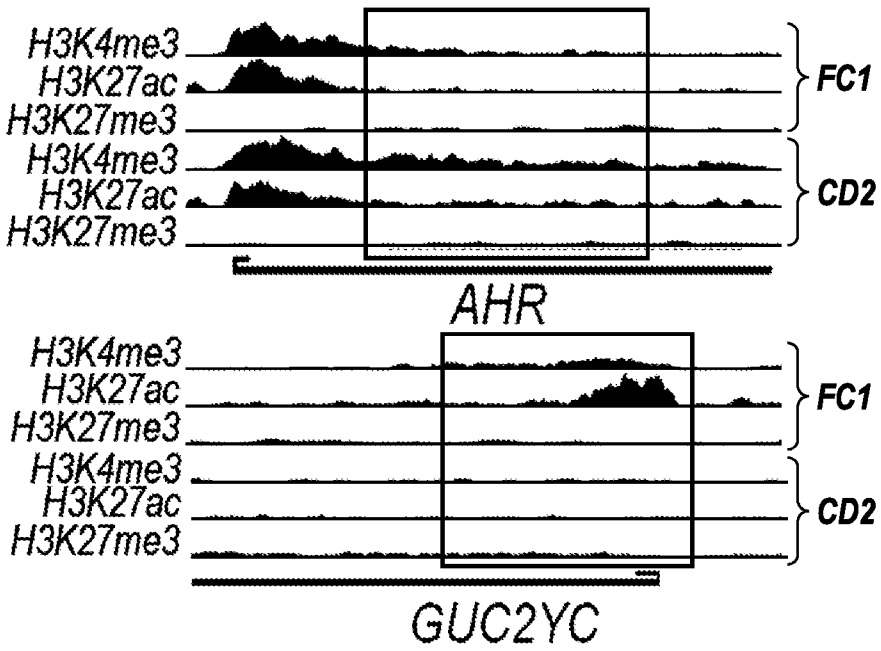


Fig. 18

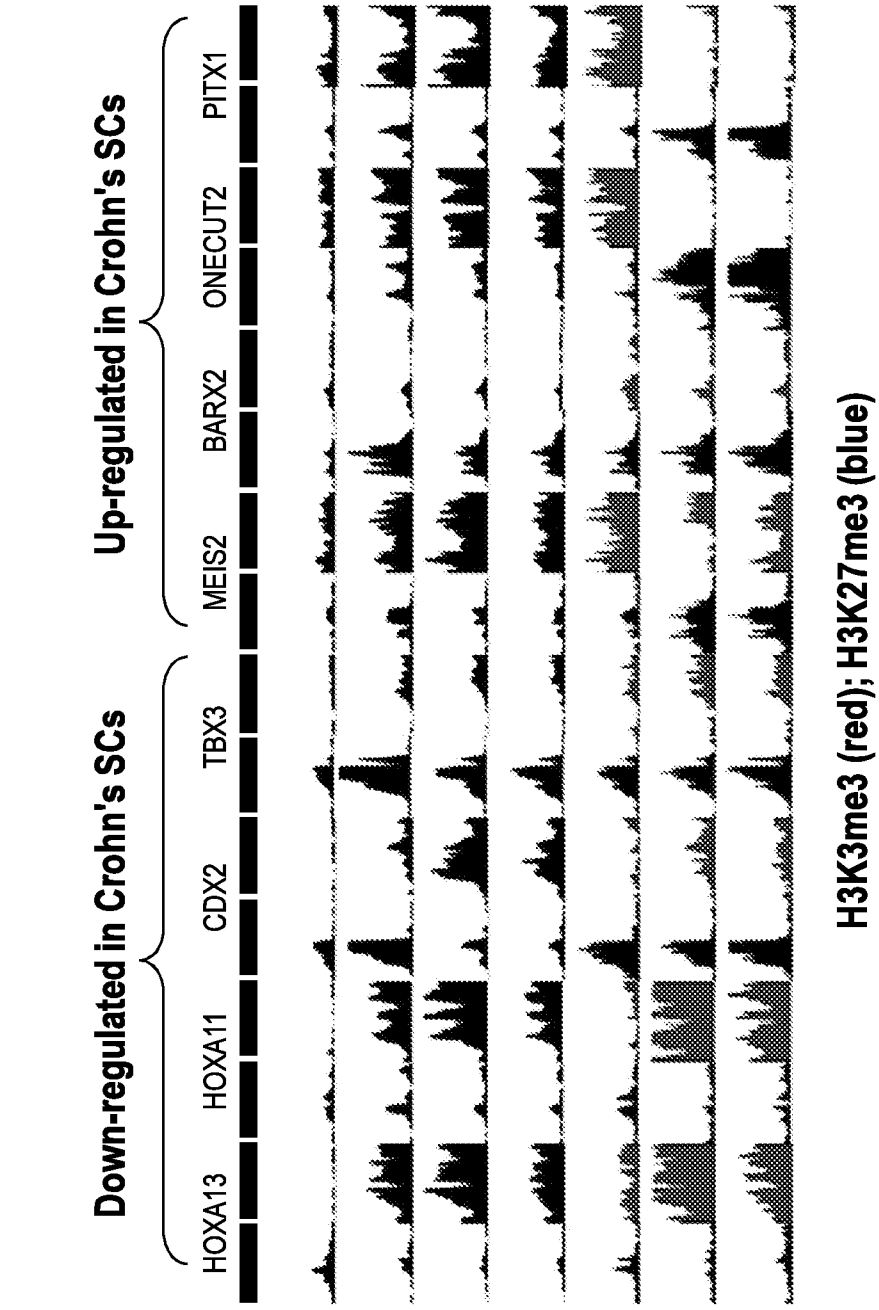

Down-regulated in Crohn's SCs
Up-regulated in Crohn's SCs
HOXA13
HOXA11
CDX2
TBX3
MEIS2
BARX2
ONECUT2
PITX1
Normal TI
Crohn's Cluster TI
Ascend colon
Duoden
Antr
H3K3me3 (red); H3K27me3 (blue)

Fig. 19A

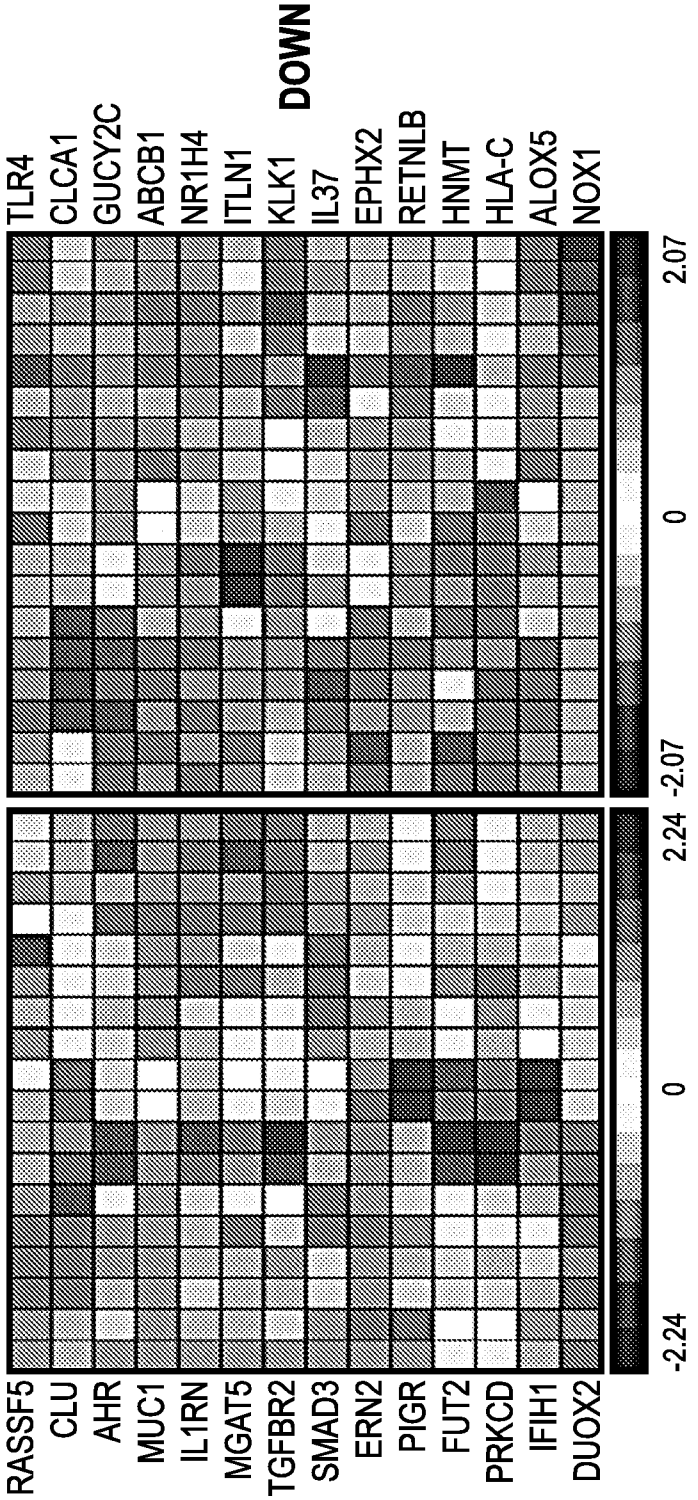
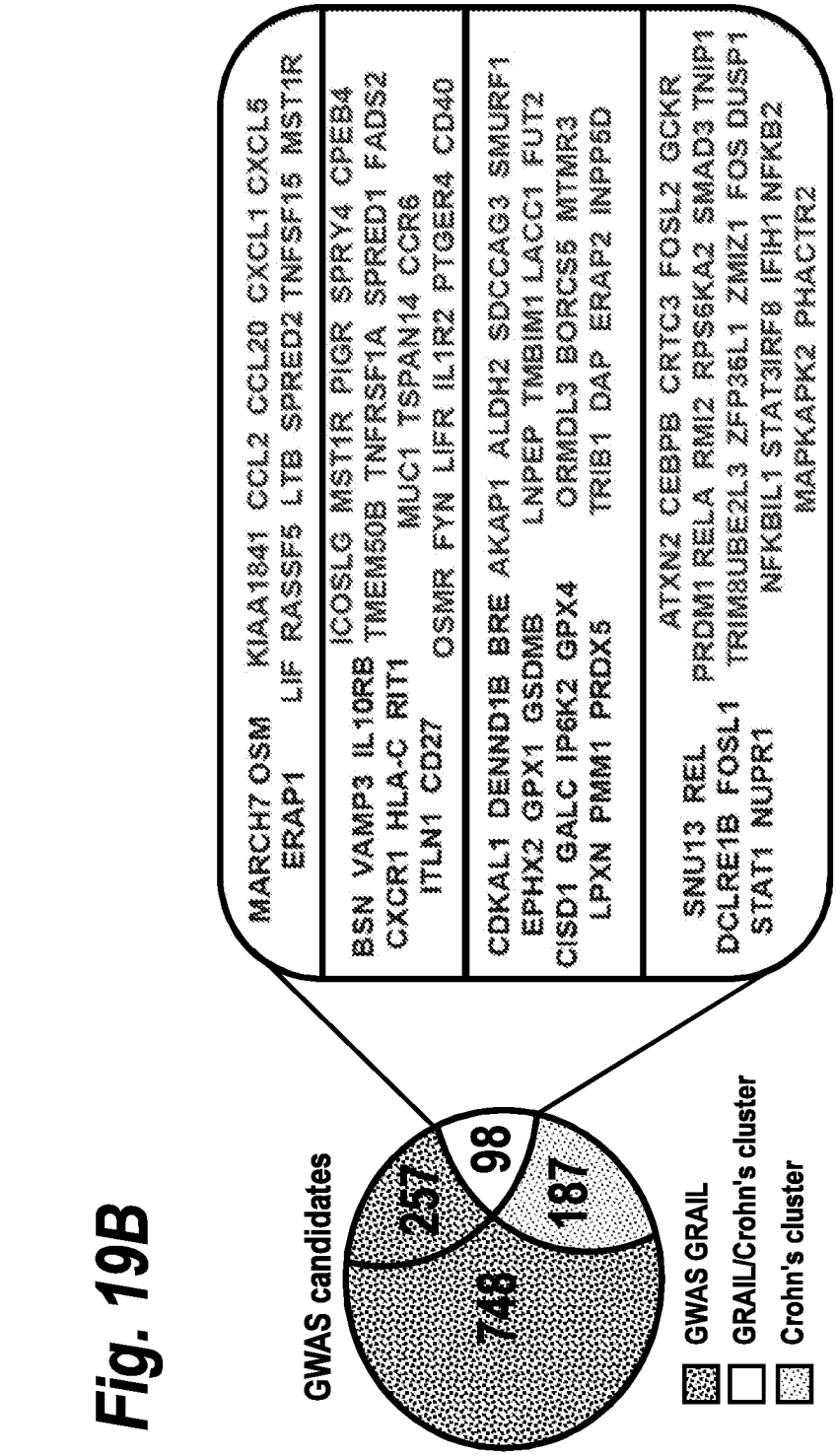

Stomach
Intestine
Colon
A B C D E F G H I
UP
RASSF5
CLU
AHR
MUC1
IL1RN
MGAT5
TGFBR2
SMAD3
ERN2
PIGR
FUT2
PRKCD
IFIH1
DUOX2
-2.24
0
2.24
DOWN
TLR4
CLCA1
GUCY2C
ABCB1
NR1H4
ITLN1
KLK1
IL37
EPHX2
RETNLB
HNMT
HLA-C
ALOX5
NOX1
-2.07
0
2.07

Fig. 19B


GWAS candidates
257
98
187
748
GWAS GRAIL
GRAIL/Crohn's cluster
Crohn's cluster
MARCH7 OSM KIAA1841 CCL2 CCL20 CXCL1 CXCL5
ERAP1 LIF RASSF5 LTB SPRED2 TNFSF15 MST1R
BSN VAMP3 IL10RB ICOSLG MST1R PIGR SPRY4 CPEB4
CXCR1 HLA-C RIT1 TMEM50B TNFRSF1A SPRED1 FADS2
ITLN1 CD27 MUC1 TSPAN14 CCR6
OSMR FYN LIFR IL1R2 PTGER4 CD40
CDKAL1 DENND1B BRE AKAP1 ALDH2 SDCCAG3 SMURF1
EPHX2 GPX1 GSDMB LNPEP TMBIM1 LACC1 FUT2
CISD1 GALC IP6K2 GPX4 ORMDL3 BORCS5 MTMR3
LPXN PMM1 PRDX5 TRIB1 DAP ERAP2 INPP5D
SNU13 REL ATXN2 CEBPB CRTC3 FOSL2 GCKR
DCLRE1B FOSL1 PRDM1 RELA RMI2 RPS6KA2 SMAD3 TNIP1
STAT1 NUPR1 TRIM8UBE2L3 ZFP36L1 ZMIZ1 FOS DUSP1
NFKBIL1 STAT3IRF8 IFIH1 NFKB2
MAPKAPK2 PHACTR2

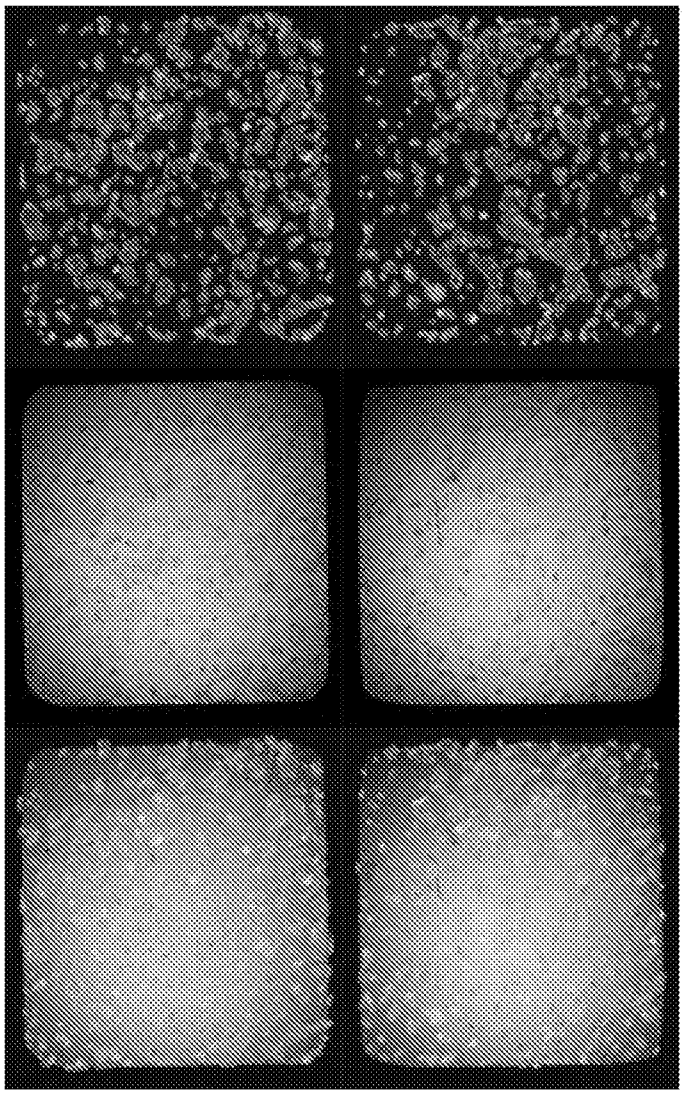


Fig. 20

Fig. 21

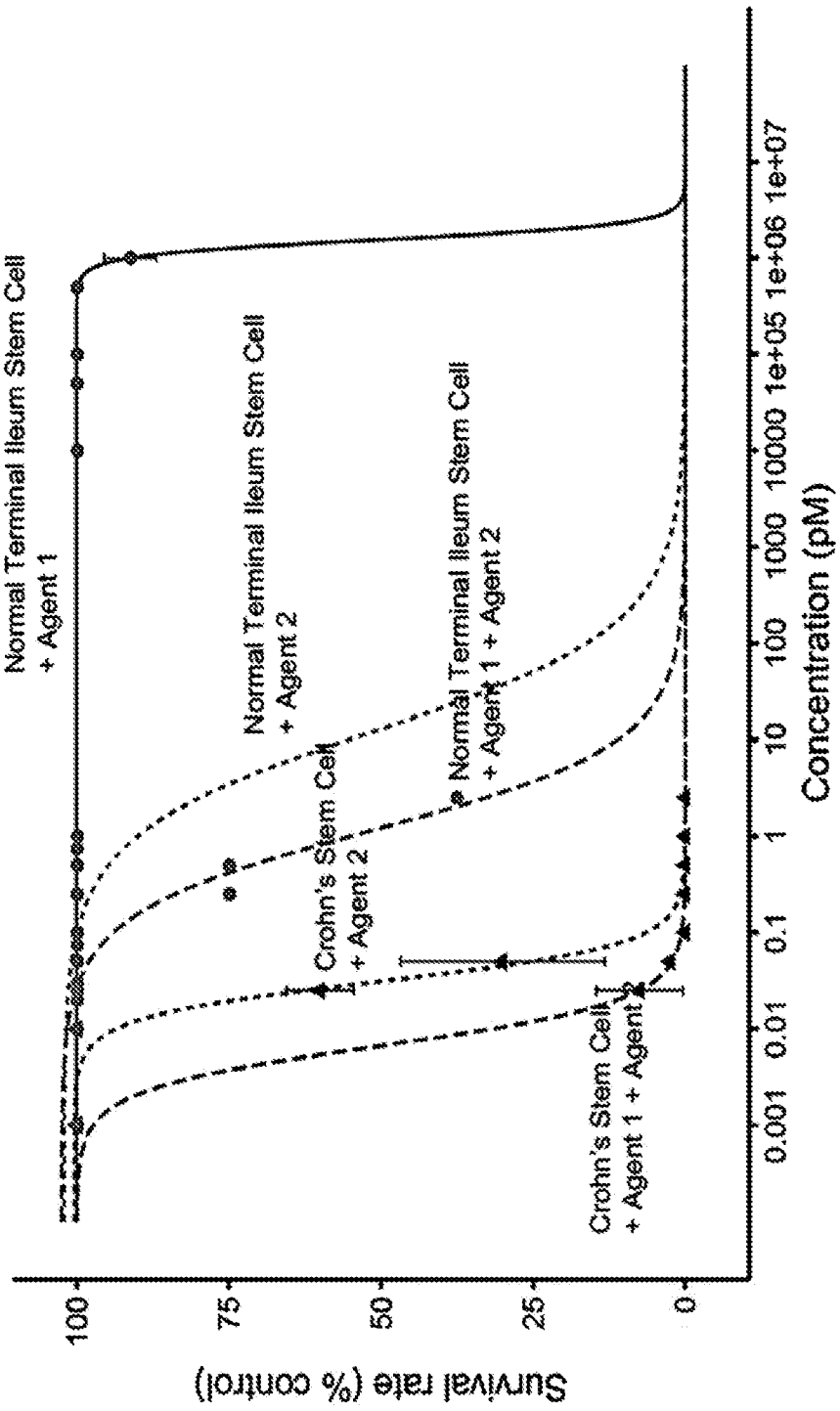

Survival rate (% control)
100
75
50
25
0
0.001
0.01
0.1
1
10
100
1000
10000
1e+05
1e+06
1e+07
Concentration (pM)
Normal Terminal Ileum Stem Cell + Agent 1
Normal Terminal Ileum Stem Cell + Agent 2
Crohn's Stem Cell + Agent 2
Normal Terminal Ileum Stem Cell + Agent 1 + Agent 2
Crohn's Stem Cell + Agent 1 + Agent 2

Fig. 22

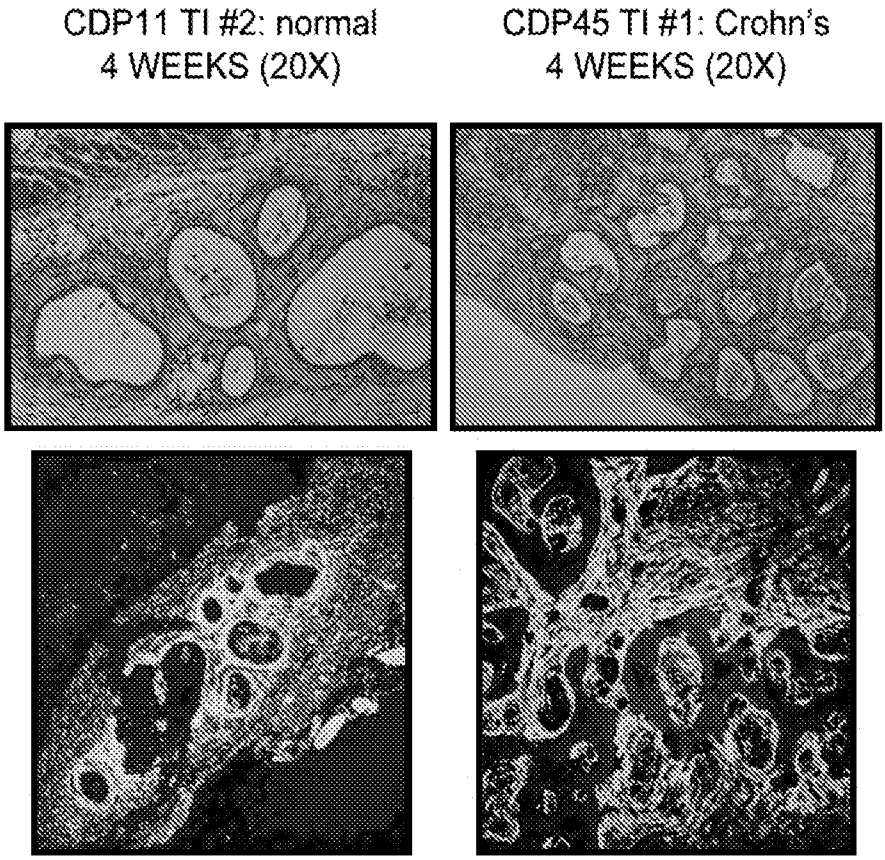
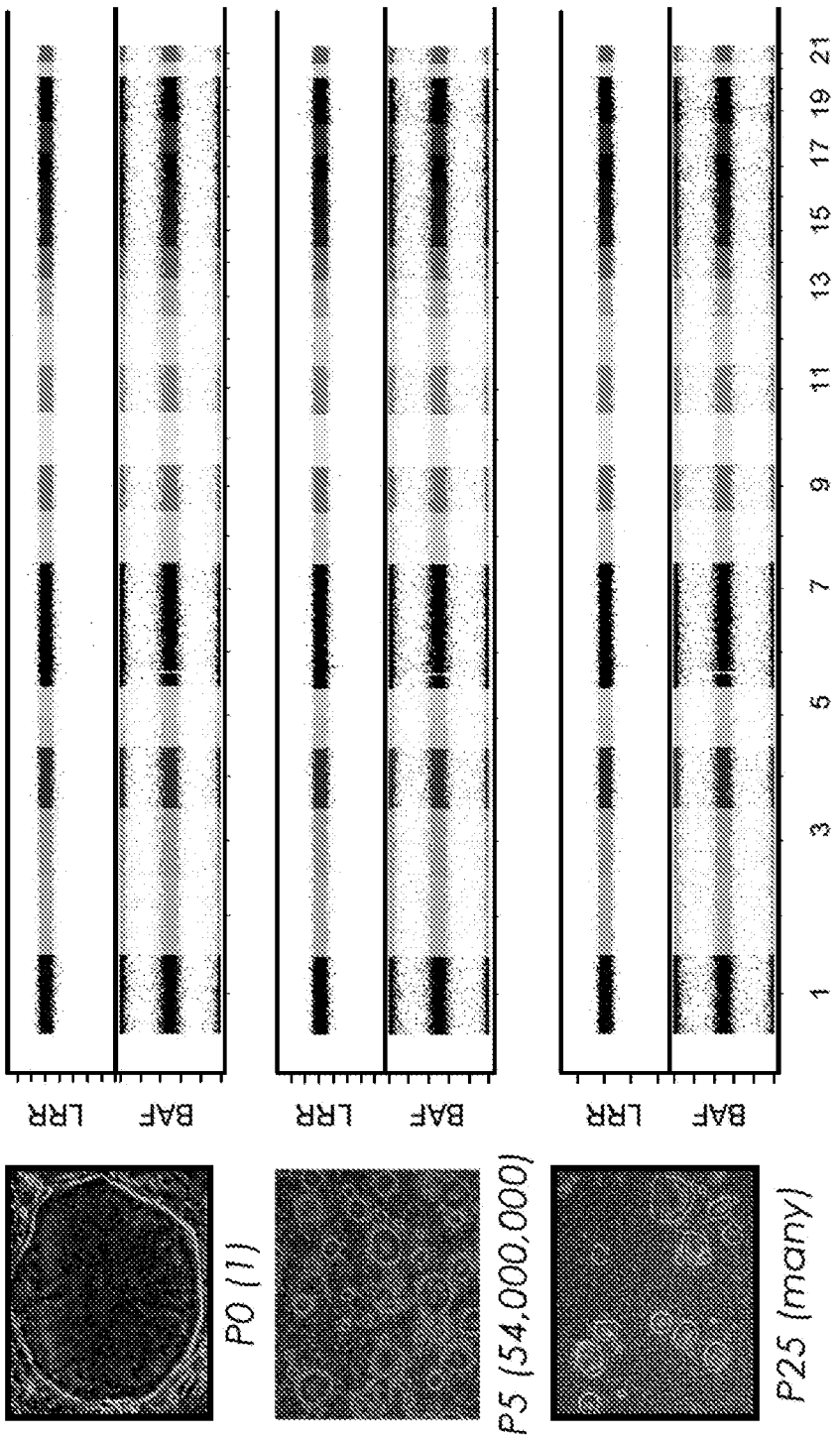


Fig. 23



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/031370

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 35/38; A61K 38/005; A61K 38/18; A61K 38/1841; A61K 2039/836; A61P 1/00 (2018.01)

CPC - A61K 35/38; A61K 38/18; A61K 38/1825; A61P 1/00; A61P 37/00; C12N 5/0679; C12N 5/068; C12N 2501/15; C12N 2501/415; C12Q 1/68; C12Q 1/6876; C12Q 2600/158; G01N 33/5073; G01N 2500/10 (2018.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2016/0237400 A1 (THE JACKSON LABORATORY) 18 August 2016 (18.08.2016) entire document	1-7, 9-11, 13-15, 20, 21 --- 12, 16-19
Y	US 2007/0140966 A1 (CHANG et al) 21 June 2007 (21.06.2007) entire document	12, 16-19
A	DOTTI et al. "Alterations in the Epithelial Stem Cell Compartment Could Contribute to Permanent Changes in the Mucosa of Patients with Ulcerative Colitis," Gut, 01 November 2016 (01.11.2016), Vol. 66, No. 12, Pgs. 2069-2079. entire document	1-7, 9-21
A	US 2016/0060594 A1 (THE JACKSON LABORATORY) 03 March 2016 (03.03.2016) entire document	1-7, 9-21
A	WANG et al. "Cloning and Variation of Ground State Intestinal Stem Cells," Nature,03 June 2015 (03.06.2015), Vol. 522, No. 7555, Pgs. 173-178. entire document	1-7, 9-21
A	US 2016/0061817 A1 (THE JACKSON LABORATORY) 03 March 2016 (03.03.2016) entire document	1-7, 9-21
A	US 2015/0044135 A1 (XIAN et al) 12 February 2015 (12.02.2015) entire document	1-7, 9-21
A	YAMAMOTO et al. "Mutational Spectrum of Barrett's Stem Cells Suggests Paths to Initiation of a Precancerous Lesion" Nature Communications, 19 January 2016 (19.01.2016), Vol. 7, No. 10380, Pgs. 1-10. entire document	1-7, 9-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 August 2018	25 SEP 2018
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/031370

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/031370

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 8
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.