CHARACTERISATION OF COLONIC EPITHELIAL STEM CELLS IN HEALTH AND INFLAMMATORY BOWEL DISEASE

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DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that the thesis entitled "Characterisation of Colonic Epithelial Stem cells in Health and Inflammatory Bowel Disease" is based on the results of the work carried out by Ms. Akila G. during the period of study under my supervision and guidance. This work or thesis has not found the basis for the award of any degree, Diploma, Associateship, Fellowship or other similar title.

The candidate independently reviewed the literature and set up and standardized the various described techniques in this thesis. She was primarily responsible for collection of biological samples and for their analysis. She has written up the work and has done the necessary statistical analysis and presentations.

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CERTIFICATE

I hereby declare that this Thesis entitled " Characterisation of Colonic Epithelial Stem cells in Health and Inflammatory Bowel Disease" is done by me under the supervision and guidance of Dr. B.S. Ramakrishna, in Wellcome Trust Research Laboratory, Christian Medical College, Vellore, is true to the best of my knowledge and belief.

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The project titled " Characterisation of Colonic epithelial Stem cells in Health and inflammatory Bowel Disease" has been reviewed by the Institutional Review Board of the Christian Medical College which considered its objective, study design and budget. This study has been approved for conduct at the Christian Medical College, Vellore under the direction of Dr. B.S. Ramakrishna and Ms. G. Akila, Department of Gastrointestinal Sciences. The IRB of Christian Medical College is registered and the institution holds FWA Number FWA00002210.

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This is to certify that the animal studies done by Akila G. as part of her PhD thesis titled "Characterization of Colonic Epithelial Stem Cells in Health and Inflammatory Bowel Disease", was approved by the Institutional Animal Ethics Committee of Christian Medical College, Vellore (IAEC Approval Number 10/2009 dated 09/10/2009).

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CONTENTS

LIST OF ABBREVIATIONS

BMP- Bone Morphogenetic Protein

BSA- Bovine Serum Albumin

cDNA- Complementary De- oxy Ribo Nucleic Acid

CD - Chron's Disease

DTT- Dithio threitol

DAB - Di Amino Benzidine

DSS - Dextran Sulphate Sodium

DMEM- Dulbeccos Modified Eagles Medium

DNA- De-oxy Ribo Nucleic Acid DAPI-

DAPI- 4,6, Diamidino -2- Phenyl Indole dihydrochloride

EGF- Epidermal Growth Factor

EDTA- Ethylene –Diamine, Tetra Acetic acid

FGF- Fibroblast Growth Factor

FITC- Fluorescein Iso Thio cyanate

FOB - Fecal Occult Blood

GE- Genomic Elimination buffer

HES-1 – Hairy and Enhancer of Split -1

ISC – Intestinal Stem Cells

IBD - Inflammatory Bowel Disease

LIM 1863 - Large Intestinal Mucosal cell line

MSC – Mesenchymal Stem Cells

PBS - Phosphate Buffered Saline

PCR- Polymerase Chain Reaction

PFA- **Paraformaldehyde**

Poly2HEMA - **Hydroxy Ethyl Methacrylate**

RNA- **Ribo Nucleic Acid**

RPMI 1640- **Rosewell Park Memorial Institute medium**

UC - **Ulcerative Colitis**

WNT- **Wingless Tail**

Y-FISH- **Fluorescence In Situ Hybridisation**

INTRODUCTION

The epithelium of the large intestine is a very rapidly replicating tissue with an enormous capacity for continuous proliferation, followed by differentiation, senescence and shedding. The proliferative capacity of the large intestinal epithelium is maintained through multipotent stem cells which are thought to be located towards the base of the colonic crypts. As yet poorly defined signals, that may include cytokines and growth factors which are secreted by the lamina propria cells or the intestinal subepithelial myofibroblasts, control the proliferation and differentiation of these epithelial stem cells (1). Stem cells of the intestinal epithelium are difficult to isolate from the whole crypt due to the lack of specific markers. Stem cells in general are characterized by asymmetrical replication with one daughter cell maintaining stem cell characteristics while the other daughter cell undergoes some differentiation or commitment towards a particular epithelial cell lineage. The daughter stem cell remains at the base or the lower third of the crypt while the other daughter cell becomes a Transit Amplifying (TA) progenitor, located in the mid of the crypt. This then leads to five important differentiated lineages of columnar cells, namely absorptive epithelial cells, mucin producing goblet cells (providing protection to the intestinal epithelial cells against the proteolytic action of the digestive enzymes), neuro- or enteroendocrine cells which secrete peptide hormones, Paneth cells that secrete a number of proteins including lysozyme, tumor necrosis factor alpha and antibacterial defensins, and the fifth cell type M (membranous or microfold) cells that help in antigen transportation and reside near the Peyer's patches. The intestinal epithelial cells after their fate are exfoliated into the intestinal lumen after a specific period of time. The intestinal stem cells replicate by a process of self renewal once every three to four days to maintain intestinal homeostasis (2) In spite of constant proliferation and self-renewal of stem cells by asymmetric division that takes place in every 3-4 days, the exact number and behavior of these

cells within the base of the crypt is still in debate (3). Primary culture of these epithelial cells are difficult to maintain for long periods of time because various systemic elements which are helpful in maintaining the intestinal tissue homeostasis in vivo are absent in the in vitro culture environment (4). Previous studies have shown that the human colonic crypts expand by crypt fission which divides to form two daughter crypts and the entire gastrointestinal tract is populated by the stem cell clones. So some evidence suggests that crypt fission is a dynamic process of clonal expansion of crypts in the normal human intestine (5). The clonogenic assay, in which a single cell can be grown as clones in soft agar, has been used as a functional assay for stem cells (6,7).

Inflammatory bowel diseases are inflammatory disorders of the gastrointestinal tract caused by an interaction between environmental factors and individuals with particular genetic make-up. The symptoms include diarrhoea, abdominal pain and weight loss. Ulcerative colitis and Crohn's disease are the two major types of inflammatory bowel diseases of the gastrointestinal tract. UC is associated with mucosal inflammation in the colon and the rectum and the onset of the disease is confirmed by the histopathological features where the infiltration of inflammatory cells such as neutrophils, monocytes and lymphocytes, macrophages and granulocytes are more prominent, loss of crypt architecture, cryptitis and crypt abscesses are seen due to the migration of polymorphonuclear neutrophils (PMNs), and lymphocytes into the lamina propria. The loss of the epithelial barrier and loss of tight junction function is one of the reasons leading to ingress of molecules from the intestinal lumen that elicit an inflammation. Several experimental animal models have been used to investigate the pathogenesis of this disease. Gene knock-out models such as IL2/IL2 receptor alpha, IL-10, T-cell receptor (TCR), trefoil factor, tumor necrosis factor (TNF) – 3'untranslated region (UTR); transgenic models

include IL-17, signal transducer and activating transcription (STAT-4), HLA B 27; spontaneous colitis models and inducible colitis models of TNBS colitis, dextran sodium sulphate (DSS) colitis and PG-PS colitis, and adoptive transfer colitis of T cell transfer induced colitis, CD45 RB transfer model. The DSS colitis model is used widely due to its high degree of reproducibility and resemblance to human UC. The mechanism of action of the DSS induced colitis in animals in still unknown but the evidence suggests that it may be due to the direct toxic effect on the epithelial cells of the basal crypts, so the numbers and the location of these cells may be varied in the diseased condition (8,9).

Immunohistochemistry is a useful technique to identify the location of antigens in the tissues and to identify expression and numbers of specific markers for proliferative and differentiated cells in healthy and diseased patients. Therefore the regulation of stem cells in normal and abnormal states can be understood. Expression of Musashi-1, which is a RNA binding protein, can be identified in normal and colitic tissue models with a different staining pattern and their distribution. Hes -1, a stem cell differentiation marker, is also associated with the differentiation of epithelial cells in the crypts (10,11). Little is known about changes to stem cells in colitis and in cancer. Cancer stem cells are thought to be present in colon cancer. In colitis, the stem cell compartment appears to be expanded when PCNA expression is examined; however PCNA expression is inconsistently increased in cancer. An increase in mutated stem cells is described in patients with ulcerative colitis and this has been thought to predispose to cancer in these patients. Stem cell based therapy is increasingly being considered in the treatment of inflammatory bowel disease, but this is predominantly based on hematopoietic and mesenchymal stem cells. (12, 13, 14, 15). The stem cell regulation is determined by the Notch signaling pathway where Hes-1 is the downstream protein of this signaling. This signaling is

involved in the epithelial cell fate decision and differentiation of the four specialized cell types in the intestine. This pathway is based on the Unitarian hypothesis where all the four differentiated epithelial lineages arise from a single stem cell (3). HES is the Hairy and Enhancer of Split gene of Drosophila of basic helix-loop-helix protein. There are 7 different subtypes of Hes, out of which Hes-1 is considered as a DNA binding transcription factor which determines cell fate decisions and lineage specification of absorptive cells significantly in intestinal epithelial tissue of mouse and humans. Hes-1 is widely upregulated in cancers particularly in ovarian cancer hence its expression is thought to be regulated during abnormal proliferation of intestinal stem cells during ligand-receptor activation of differentiation process of epithelial cells (16). The studies described in this thesis were designed to characterize epithelial stem cell characteristics and distribution (17) in the normal colon and its alteration in inflammation in an experimental animal model as well as human colitis.

AIMS AND OBJECTIVES

OBJECTIVES:

1. To isolate and characterize proliferative cells from the stem cell compartment of the colonic epithelium.

2. To determine whether the above proliferative epithelial cells can ameliorate experimental colitis in mice.

3. To determine whether there is an alteration in epithelial stem cell numbers and distribution in the colonic mucosa of patients with inflammatory bowel disease.

REVIEW OF LITERATURES

REVIEW OF THE LITERATURE

1. Stem cells
2. Intestinal epithelial stem cells
3. Inflammatory bowel disease
4. Stem cells in IBD
5. Stem cells in other colonic diseases

1. **STEM CELLS:**

Stem cells have the enormous potential to form diverse cell types in the body during the early life and growth phase. In almost all tissues they act as an internal repair system to some extent with unlimited proliferation and to replace the old cells throughout the life of the organism. When the stem cell divides either two of the cells exists as stem cells called as symmetric division or one cell remain as stem cell and the other becomes more specialized cell type either a muscle cell, or blood cell or a brain cell. Stem cells can be differentiated from other cell types by two important characteristic features. The first is by their self renewing capacity even after long periods of their inactivity. Secondly, under certain conditions like physiologic or experimental induction lead them to become tissue or organ specific cells with special functions. Certain organs such as gut and the bone marrow have the constant proliferation of stem cells to repair or replace the worn out tissues, but in other organs such as heart and pancreas, stem cells only divide under special conditions. Stem cells play a vital role in living organisms for many reasons, for example in 3-5 days old embryo which is called as blastocyst, the inner cell mass has the capability of giving rise to the entire body of the organism such as heart, lung , skin, sperm, eggs and other tissues. But in adult tissues, the stem cells help in replacement during injury or during disease conditions especially in bone marrow, muscle and brain. Due to their regenerative abilities, stem cells are used as potential therapeutics for treating diseases such as diabetes and heart disease. (19,20,21,22)

EMBRYONIC STEM CELLS:

Embryonic stem cells are derived from embryos which are developed from eggs that have been fertilized *in vitro* that are donated for research purpose. Human embryonic stem cells are cultured in a dish with appropriate culture conditions which are coated with mouse skin

fibroblasts treated with an antibiotic so that the cells will not grow but act as a feeder layer for the embryonic stem cells to proliferate indefinitely. Embryonic stem cells which are cultured continuously for a prolonged period of time without differentiation may be pluripotent in nature. Two of the transcription factors which are produced by embryonic stem cells are Oct-4 and Nanog which helps these cells to maintain an undifferentiated state for a long time and capable of self-renewal.(23,24,25). There are various tests used to determine whether these stem cells maintain pluripotency. One is to make the cells to differentiate spontaneously in culture. Another is to manipulate the cells so that they will differentiate to form three germ layers and injecting these cells into an immune compromised mouse to test the formation of benign tumor called teratoma. These teratomas contain a complex of differentiated cell types which is the indication that the embryonic stem cells has the capability of differentiating into multiple cell types. The differentiation occurs spontaneously when these cells clumps together and forms embryoid bodies.(26,27).

ADULT STEM CELLS:

Adult stem cells are also undifferentiated cells which are found along with the differentiated cells in the tissues or organs, renew themselves and give rise to all of the specialized cell types upon differentiation of the tissue or organ (28). The major role of adult stem cells is to maintain the stem cell numbers and to repair the tissue during injury or any damage. Adult stem cells can be used for transplantation studies if they could be maintained in a undifferentiated state in an *in vitro* culture for a long time. These stem cells are found in almost all tissues like brain, bone marrow, peripheral blood, blood vessels, skin, skeletal muscle, teeth, heart, gut, liver, ovarian epithelium and testis and they are reside in a specialized place called the stem cell niche. Each tissue is accompanied with stem cell compartment and differentiated compartment in which stem

cells alone will get growth factors and signaling molecules from the surrounding microenvironment and maintains the symbiotic relationship between the niche and the surrounding cells. These stem cells may be in quiescent state even for a long period of time until they are needed for tissue maintenance or under abnormal conditions to safeguard the tissue functionality. (29-41).

IDENTIFICATION OF ADULT STEM CELLS:

The methods which are used to identify these stem cells are by labeling the cells in a living tissue with specific molecular markers and then determine the cell types they generate, isolating cells from a live animal and culture in *invitro* conditions with labeling, third possibility is that the cultured cells can be transplanted into mismatched animal to determine whether the cells have the ability to repopulate the tissue of origin.(42, 43).

DIFFERENTIATION PATHWAYS OF ADULT STEM CELLS:

Various adult stem cells give rise to different differentiation pathways according to the nature of the tissue. For example, haematopoietic stem cells give rise to all the types of blood cells such as red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophils, eosinophils, monocytes and macrophages. Neural stem cells produce three major cell types namely nerve cells and two categories of non-neuronal cells astrocytes and oligodendrocytes. Bone cells, cartilage cells, fat cells and other kinds of cells such as connective tissue all arise from mesenchymal stem cells. Absorptive cells, goblet cells, Paneth cells, and enteroendocrine cells are the differentiated cells from the epithelial stem cells which reside at the base of the crypts. Skin stem cells occur at the base of the epidermis and at the base of the hair follicles whereby protective layer of the skin is formed called keratinocytes. (44,45).

USES OF HUMAN STEM CELLS:

There are different ways by which human stem cells can be used in the treatment of diseases. Studies about the human embryonic stem cells provide information about the complex events that give rise to organ development. Some of the serious medical conditions such as cancer and genetic defects are due to abnormal cell division and differentiation. Human stem cells can also be used to test different kinds of drugs. New therapeutic agents can be identified for safety on differentiated cells which are generated from pluripotent cell lines. Tissue specific pluripotent stem cells would be used to test wide variety of drugs. Human stem cells can be used instead of transplanting organs and tissues due to lack of availability of organ donors. Till now stem cells have been used to treat Alzheimer's diseases, spinal cord injury, stroke, burns, heart disease, diabetes, osteoarthritis, rheumatoid arthritis(46,47).

2. INTESTINAL EPITHELIAL STEM CELLS

The general organization of the adult colonic epithelium is quite similar with that of the small intestine with the exception that there are no villi in the colon. The gastrointestinal tract is the primary example for the continuous turnover of the cells because cells which are extinct due to injury or due to irradiation or by their terminal differentiation should be replaced to maintain the normal functionality of the tissue. The rate of cell replacement should be matched to the rate of cell loss so that the tissue can able to maintain intestinal homeostasis under any conditions. The four differentiated cells of the colon are shed into the intestinal lumen and they are replaced continuously by the transit amplifying cells which are considered as the progeny of stem cells residing at the base of the crypt which occupies first 3 tiers in the whole crypt. The intestinal epithelial stem cells can be defined as undifferentiated cells which are capable of unlimited proliferation, ability for self-maintenance, maintenance of pluripotency (giving rise to a variety

of cell lineages), and the capability to regenerate the entire epithelium during injury (48). The time taken for cell division in the progenitor zone is estimated to be approximately 30-33 hours (49-52). But the true epithelial stem cell takes more time for its cell cycle when compared to the transit amplifying cells. Identification of this pluripotent stem cell in section may be achieved through the immunohistochemical markers such as Musashi-1, while the functional characteristics of the stem cells and the progenitors have been inferred by experimental analysis of the cell lineages they produce. Numerous studies have implicated that every single adult crypt is monoclonal, that is genetically derived from a single stem cell. These results came from number of different experimental systems in which histochemical markers were used to identify the genetic origins or tag lineages of cells within the crypt(53,54,55). The model systems used were: allophenic tetraparental mice, use of X chromosome-linked enzyme markers, *in vivo* mutagenesis in mice heterogenous for cell surface lectin markers and studies tracing age-dependent extinction of transgene expression in the colon. In all these systems, the behavior of the crypt is like as if all of its cells are derived from a single multipotent stem cell (56,-59). The concept of the stem cell niche is now widely studied. The niche is the specialised place with physical and structural environment in which the stem cell reside and it was first proposed by Schofield. (60).The structure of the epithelial stem cell niche in the colon is formed during intestinal development by the invagination of the intervillus epithelium into the surrounding mesenchyme. Several cell types are present in the niche namely intraepithelial lymphocytes, blood vessels, enteric neurons and pericryptal fibroblasts. Additionally, secretion of growth factors such as hepatocyte growth factor, transforming growth factor-beta, various fibroblast growth factors, and some other growth factors secreted by the intestinal subepithelial myofibroblasts play a vital role in the differentiation of the epithelial cells that express receptors

for these factors (61). Even in the absence of stem cells the niche exists, and this is confirmed by experiments in which targeted disruption of the T–cell Factor (TCF-4) gene resulted in the depletion of proliferating cell population but the pericyprtal mesenchymal components of the niche remained. Likewise, the lethal dose of radiation will destroy the epithelial component of the intestinal niche, but if one single stem cell escapes this radiation injury, the remaining mesenchyme forms the entire crypt (62).

LOCATION:

The exact number and location of stem cells in the small intestine and the colonic crypts may vary. This number has been determined by cell proliferation studies and mathematical modelling by the incorporation of Htdr to the nuclei of DNA synthesizing strands during proliferation and BrDU labeling studies by tissue injury by radiation exposure. There are two theories that exist for the location of stem cells. The first one states that crypts are polyclonal in nature during embryonic and neonatal development and that during the development of an organism, they transforms into monoclonal adult crypts (63). Mutational and gene induction studies supports this theory because under normal tissue homeostasis, the stem cell number in the small intestine and the colon ranges from 4-6, and in the large intestine it appears to be present in the very base of each crypt and just above the Paneth cells. There are a number of studies pursued for the location, their numbers, measurement of kinetics proliferation and their mode of lineage commitment of stem cells in the large intestine in models such as wild-type, chimeric, and transgenic mice. The normal functions of the stem cells are maintaining pluripotency when they are self renewing by themselves and be in quiescent state under normal physiological conditions(64,65). So identification of these stem cells and their location requires particular markers with a specific function. Moreover integrins and adherens junctions play a vital role in

maintaining the location and proliferative nature of the epithelial cells. Stem cells divide asymmetrically with retention of one stem cell located in the niche. So cells which undergo apoptosis do not have the capability of repairing mechanisms and die in the intestinal lumen due to radiation injury and during extinction of these stem cells even if one progenitor escapes radiation treatment, can repopulate the entire crypt in two to three days. There were studies demonstrating about the permanent residence of stem cells in the adult intestinal crypts by labeling the stem cells with two different types of markers in order to ensure which retains old and new strands. Therefore ^{3}H thymidine was labeled to stem cells either during irradiation or during neonatal life confirms that protective mechanisms against DNA-replication induced errors ensures that stem cells selectively retains old DNA template strands whereas newly synthesized strand which was labeled with BrdU (analog of thymidine) segregate to the TA cells so they were not retained. This study concluded that stem cells are located at the base of the crypt and at the position 3-4 in the small intestine. To further demonstrate that these cells retain clonogenic potential even after high dosage of irradiation and cytotoxic insult, initially for low dose of γ irradiation (1Gy) cells which were situated at the base of the small intestinal crypts first underwent apoptosis and these cells might be at the initial proliferative stage or might be the true small intestinal stem cells and were readily apoptosed instead of repairing by themselves so that the entire crypt would not be repopulated with the mutated clone. Then the administration of second higher dose of radiation (< 9 Gy), revealed the existence of second tier of cells which comprised of 6 clonogenic cells and these cells would usually be the daughter cells or progenitor cells of the stem cells which were migrating towards the upper surface of the crypt epithelium. These cells retained stem cell properties even during their early lineage commitment. Radiation treatment of even higher dose of > 9 Gy expressed additional third tier of stem cell region with

24 stem cells which has the high DNA repairing mechanisms so they have the greater capability of radioresistance. This study explained about the position of stem cells at 2-7 in the small intestine whereas in the large intestine similar experiments carried out showed that there are also small number of stem cells present in the region of four to six cells with same up to 36 clonogenic cells in total. In any case, due to this if all the true stem cells are destroyed and if one stem cell or the daughter cell was more radioresistant it will have the ability to form the whole crypt adapting the stem cell functions which they already acquired from the stem cell compartment. The remaining cells approximately 114 cells either in the small intestine or in the colon which has high proliferation rate, situated above this three tier hierarchial region does not have any clonogenic potential or stem cell properties. Thus the exact location of stem cells in the small intestine and in the colon of both rodents and humans were revealed. But it is not clear whether the third tier of cells undergoes differentiation into four types of epithelial lineages or the cells from the bottom of the crypt helps in differentiation(66-70).

FATE:

It is well understood that stem cells gives rise to their immediate descendants called as transit amplifying progenitor or daughter cells which in turn produces two types of intestinal lineages such as absorptive and secretory lineages. Each has different cell types specifying particular function. It is not clearly known whether stem cell division directly yield cells which are committed to differentiation or the transit amplifying cells through the interaction with other cells forms four types of epithelial cells. The unitarian hypothesis defines that all the epithelial lineage cells from the gastrointestinal epithelium are derived from a single stem cell which is located at the base of the crypt even though the experimental evidence suggests that 4-6 stem cells are present in each crypt base. There are four principal epithelial lineages present in the

gastrointestinal tract in which each of these cells differ morphologically and functionally. Columnar cells termed as enterocytes in the small intestine and colonocytes in the large intestine is the major epithelial cells in the mucosa. Mucin secreting cells are called as goblet cells in both the small and large intestine, endocrine and Paneth cells function in peptide hormone secretion and expression of specific proteins(71). There are two mechanisms that explain the fate of intestinal stem cells via Notch signaling and by genetic studies in chimeric mice(72).

NOTCH SIGNALLING PATHWAY:

The Notch signaling pathway regulates epithelial stem cell fate and differentiation of four specialized cell types of the intestinal epithelium . It supports the Unitarian hypothesis which states that all of the epithelial cells in the intestine were originated from a common stem cell. This molecular pathway explains that while there is an increased expression of Hes-1, the notch protein (Hairy and Enhancer of Split-1) downregulates the transcription of Math-1 gene through its transcriptional repressor Hes-1. So the genes which are committed to become goblet, Paneth and enteroendocrine cells turn into absorptive cells. Similarly increased expression of Notch levels increases its ligand Delta which blocks Hes-1 and allows Math-1 expression, then differentiation into Paneth, goblet and enteroendocrine cells results. Experiments with Hes-1 knockout mice proved this, with low numbers of absorptive cells and greater numbers of specialised cells (72,73,74).

SIGNALLING IN STEM CELLS:

TCF-4 AND THE WNT SIGNALLING PATHWAY:

Wnt are the group of proteins of secretory type, small signaling molecules which are known to regulate embryonic development, tissue morphogenesis, and maintenance of adult tissue homeostasis(75). Previous studies have shown that wnt has a critical role in the regulation of crypt epithelial stem cell proliferation during normal embryonic development and in tissue homeostasis. The best example for wnt signaling is shown in mice which lack TCF-4 (Transcription factor-4) which is the target gene of wnt. These mice do not have proliferative stem cells of the intestine in the intervillus epithelium from embryonic day 16.5. So these mice could not form nascent crypts and die shortly after birth (62).

MECHANISM OF ACTION OF WNT SIGNALLING:

The cascade of Wnt signaling occurs intracellularly by the activation of Beta catenin/TCF-4 . The primary receptors of wnts are the Frizzled family of proteins. But there is an additional requirement of transmembrane molecule of the low-density lipoprotein (LDL) receptor-related protein (LRP) family, with which it can form a trimeric receptor complex that transduces the wnt signal to intracellular proteins. Initially wnt binds to the Frizzled/LRP receptor which activates the complex and causes Axin to bind to LRP with subsequent stabilization of beta-catenin. The beta-catenin is stabilized in the cytoplasm and translocated into the nucleus where it complexes with the TCF/LEF DNA-binding proteins, converting the TCF complex from a transcriptional repressor to an activator of gene transcription. But in the absence of Wnt signaling, cytoplasmic Beta catenin complexes with Axin and adenomatous polyposis coli (APC), and gets phosphorylated by the serine/threonine kinases glycogen synthase kinase-3 (GSK-3) and casein

kinase 1 alpha. Axin and APC provides scaffolding for the interaction between the kinases and Beta-catenin. Beta-catenin is ubiquitinated and targeted for degradation by the proteosomal pathway after phosphorylation (75-88).

WNT IN EPITHELIAL MORPHOGENESIS:

Van Noort et al., through experiments in fetal intestine, proved the presence of unphosphorylated nuclear beta-catenin in the undifferentiated villus epithelium, which confirms that the activation of the beta-catenin/Tcf signaling pathway occurs as early as E16.5 in the fetal homolog of the adult crypt. This supports the role of the beta-catenin/Tcf family in the regulation of epithelial morphogenesis (89-91) .

WNT ACTIVATION BY EXTRACELLULAR SIGNALS:

The Wnt family of ligands helps in the extracellular signals that triggers the activation of beta catenin/TCF 4 intermediate signaling pathways but the mechanism of action of this extracellular signaling cascade is still unknown. FGF regulated Tcf/beta-catenin mediated transcriptional activity in endothelial cells is the best example of this extracellular mechanism. There is a possibility for this mechanism to occur in intestinal epithelial cells, as previous studies indicate that FGF receptor 3 mediates signaling events in intestinal mucosal development. FGFR-3 expression was observed in the intervillus epithelium and the stem cell region of newly forming crypts during its morphogenesis. There was a depletion of crypts and the size of the epithelial stem cell population in the intestine of suckling FGFR-3 null mice. Some experiments suggests that stimulation of CaCo2 intestinal cell line by FGFR-3 ligands or transfection with a active form of FGFR-3 induced upregulation of Tcf-4 mediated transcriptional activity in these cells.

Thus these studies showed that regulation of intestinal stem cell dynamics by the Tcf/Beta catenin family involves diverse extracellular signaling events(92-95).

NOTCH SIGNALLING PATHWAY:

There are numerous studies which explain the role of the notch signaling pathway in directing cell lineage decisions of epithelial cell progenitors in the intestinal crypts.(72,73 74), . This pathway is observed in almost all tissues and cells including T and B cells. In the vertebrate intestine four receptors (Notch 1-4) and five ligands have been identified. (Delta 1, Delta 3, Delta 4, Jagged 1 and Jagged 2). The translocation of notch fragments of the intracellular domain of notch protein into the nucleus occurs once the notch receptor gets activated, the truncated notch activates the transcription factor Su(H) (suppressor of Hairless) in the nucleus which then binds to the regulatory sequences in downstream target genes such as hairy/enhancer of split (Hes). The downstream effects of notch signaling result in modulation of proliferation, either inhibition or induction of differentiation, or inhibition of apoptosis(96-98).

MATH-1 is a basic Helix-loop-helix transcription factor that is required for secretory cell fate specification and it is a downstream effector of notch signaling. The Math-1 null mice shows normal architecture of crypt villus pattern but the villi are populated only by enterocytes and all the secretory epithelial cell types were absent. But Hes-1 leads to the differentiation of absorptive enterocyte lineage since Hes-1 null mice shows increased expression of enteroendocrine cell markers and increased numbers of goblet cell. Hes-1 exerts a negative influence on Math-1 to ensure that the exact number of secretory cells and enterocytes is generated (99).

HEDGEHOG SIGNALING PATHWAY:

Hh signaling is responsible for the developmental patterning of the gastrointestinal tract (100, 101). During the development of the intestine this pathway regulates anterior-posterior and axial patterning and it is involved in the regulation of proliferation of intestinal stem cells and the progenitors to differentiate(102,-105) . There are three members of Hh family: Sonic Hedgehog, Indian Hedgehog and Desert Hedgehog. Out of these three Shh and Ihh are expressed in the intestine. Patched (Ptch) and Smoothened (Smo) are the two proteins forms the receptor for Shh (106, 107).

Hh signaling plays a distinguished role in the colon when compared to small Intestine. Ihh is expressed in the wild type mouse differentiated colonic epithelial cells at E16.5 and it has a complex of a monolayer of polarized epithelial cells with well organized crypt structure with the proliferating cells situated at the crypt base. The cells which were unable to proliferate were located in the cuff region. In contrast with the wild type mouse, Ihh null mice had a multilayered epithelium composed of only proliferating cells and the crypts were absent suggesting that the Ihh signaling provides the signals which are required to form nascent crypts and eventual formation of differentiated cells in the fetal colon. This hypothesis has been confimed by inhibiting the Hh signaling *in vivo*. Further experiments with adult rats which were treated with cyclopamine, an inhibitor of Hh signaling that binds to Smo receptor, presented notable histologic changes, along with the loss of markers of mature differentiated colonocytes such as villin (specific for microvilli) and carbonic anhydrase IV (a brush border enzyme found in differentiated colonocytes.(104).

BONE MORPHOGENETIC PROTEIN SIGNALLING PATHWAY:

BMPs are secreted signaling molecules which are of mesenchymal origin and are members of the TGF beta superfamily (108). This pathway is mainly involved in gut development and in the maintenance of adult intestinal homeostasis and plays a major role in the formation of the intestinal stem cell niche (108-110) . The mechanism of action takes place by binding to the specific receptors with serine-threonine kinase activity for the signal transduction to the nucleus through nuclear binding proteins namely the shads(108). BMP 4 is mainly responsible for shaping up the stem cell niche by restricting the topographic location of nascent crypts by *in vivo* inhibition of BMP function. The BMP pathway serves as an interface between canonical Wnt signaling pathway and the Hh pathway. BMP is the target gene for wnt pathway which helps in maintaining and regulating the stem and progenitor cells of the crypt epithelium. BMP4 along with other mediators of Hh signaling are expressed by mesenchymal cells and constitutes a network port between the epithelium and the mesenchyme in the stem cell niche. The interaction of these two pathways is achieved during late intestinal development. Moreover, this cooperation also serves as a rate limiting step to Wnt/Beta catenin/Tcf signalling to the stem cell niche. Based on these studies He et al., proposed that action of stem cell self-renewal depends on two signals one is wnt signal and by transient suppression of BMP signal via the expression of BMP inhibitor Noggin (111-114) .

ULCERATIVE COLITIS

Inflammatory bowel disease is a chronic disorder characterized by chronic inflammation occurring in any region of the gastrointestinal tract and of unknown etiology. Ulcerative colitis and Crohn's disease are the two major forms of inflammatory bowel disease and can be diagnosed by clinical, endoscopic and histological characteristics(115-117). Ulcerative colitis is largely confined to the colonic mucosa and colectomy is a cure for this disease whereas in Crohn's disease, the entire thickness of the wall of the intestine is inflamed (transmural inflammation) from mucosa to serosa and resection of the affected part is not often curative because of recurrence. Even though there are distinct patterns of disease distribution there is no specific single finding sufficient to diagnose either ulcerative colitis or Crohn's disease. Patients suffering from IBD who have disease characterstics of both diseases and cannot be classified as one of the other have indeterminate colitis (118). Even though there are similarities in clinical presentation and mild variation in histological findings, Crohn's disease resembles experimental T-helper-1 cell mediated colitis, whereas ulcerative colitis is most exactly similar to experimental T-helper-2 mediated colitis with the shared genetic background, and absence of standard etiologic agents or specific markers, these two diseases shows similar clinical presentation with diverse causes and more than two diseases can be considered in the name of inflammatory bowel disease. Even though there has been much progress seen in histopathological diagnosis and management of IBD, there are no perfect therapies yet available (119).

The term UC was first coined by Dr. Samuel Wilks in 1859 who initially named it as idiopathic colitis and observed it as different from common bacillary dysentery. In 1909, Hawkins explained about the nature of this disease which is chronic and relapsing during its

course with bleeding often occurring even in the presence of constipation. In the same year Sir Arthur Hurst demonstrated sigmoidoscopic appearances of UC and its distinction from bacillary dysentery. The primary cause of UC may be due to infectious agents or psychosomatic origin. The etiology of UC is still in debate but there are many factors involved for the cause of the disease like genetic, immunologic and environmental factors. Currently, the patients with UC show a broad spectrum of associated diseases and diverse extraintestinal manifestations(120-123).

ETIOLOGY AND PATHOGENESIS:

The etiology of UC is still in debate but there are many factors involved for the cause of the disease like genetic, immunologic and environmental factors. Variations in enteric immune response in genetically predisposed persons is the main root cause for acute and chronic inflammation and the pathologic feature of mucosal damage. The specific antigens for the inflammatory actions have not yet been confirmed but evidence suggests that it may be due to pathogenic and commensal microorganisms, metabolic by-products of these agents and normal epithelial structure (119).

Genetics:

Family history is one among the major causes for the development of this disease. This has been recognized for many years but first degree relatives are more affected than second degree relatives and more commonly it is shared among siblings. The strongest evidence of this genetic susceptibility of UC comes from twin pair studies from large European population suggests that around 6%-16% of monozygotic twin pairs are concordant for development of UC when compared to 0% to 5% dizygotic twins. Because the concordant values are much lower than

Crohn's disease, genetic determinants play a less significant role in UC than Crohn's disease. A very small number of twin pairs show UC in one twin and Crohn's disease in the other twin. It seems that genetic mutations also play a major role in the onset of this disease(124-127).

Environmental factors:

It has been the universal truth that the root cause of IBD is due to continuous antigenic stimulation by commensal enteric bacteria, fungi or viruses which leads to chronic disease in genetically susceptible hosts who already had defects in mucosal barrier function, microbial killing or immune regulation. Although several microorganisms are prone to induce this disease till now there is no specific organism that has been isolated consistently from UC patients. Therefore it is difficult to interpret that a single organism is responsible for this disease. The UC and Crohn's disease affects most probably terminal ileum and the colon where there is a high colonization of the bacteria. Previous studies have proved that intestinal inflammation can be prevented even in genetically susceptible rodents grown in a germ free environment(128,129,130). Other studies illustrates that like human gut, rodent gut inflammation can be treated with antibiotics and probiotics. Recent studies on the human gastrointestinal microbiome which speculates about the numbers of bacterial microflora present in the normal human adult gut gives the total percentage of bacteria and some general mechanisms reveals how the components of this bacterial microbiome affects the intestine which leads to chronic inflammation. Initially intestinal inflammation is produced by microbial adherence and gradually invading the epithelial cells, so that proinflammatory cytokines production were down-regulated or by producing cytotoxins. Secondly due to the imbalance in the protective and harmful bacteria leads to this disease. The other ways that bacteria could affect the IBD infected persons is by host itself in genetic defects of microbial killing, or impaired

mucosal barrier function can lead to immune hyper-responsiveness to intestinal bacteria(131,132,133,134).

Epithelial cells and their role in UC

Epithelial cells play a vital role in mucosal immune barrier function. Colonocytes can act as antigen presenting cells since it expresses MHC class II antigen, and it expresses cytokine receptors and secretes various cytokines and chemokines, and express leukocyte adhesion molecules, so any alterations in these results in UC. Another important feature is patients with UC have increased turnover of epithelial cells and other abnormalities including decreased metabolism of short chain fatty acids especially butyrate, due to decreased concentrations of Firmicutes (specifically Lachnospiraceae) by 300 fold and Bacteriodes by 50 fold in the gut flora, abnormal mucosal permeability, altered composition of glycoprotein mucus produced by the colonic epithelium. The mucosal layer of the UC patients appears to be thinner than in control subjects which paves the way for the bacteria to get adherent in both mucosal layer and at the epithelial surface. Some animal models of colitis have also suggested the role of epithelial cells in the pathogenesis of IBD by the disruption of colonic epithelium(135-146).

Psychogenic factors:

Psychogenic factors were said to be involved in the pathogenesis of UC initially, but after the discovery of immunotherapy regimens like glucocorticoids for the treatment of UC turned the focus on immunologic aspects for the pathogenesis of this disease in 1950, so these factors had less attention in UC. Experimental evidence proved that induction of stress prior to pro-inflammatory stimuli increases the inflammation in rats, but the levels of vasopressin or corticotrophin releasing factor were not involved during stress but the intestinal permeability was

directly up-regulated due to the action of cholinergic nerves, to induce intestinal inflammation in this situation(147,148,149).

Pathology of ulcerative colitis:

Patients with ulcerative proctitis have the disease confined to the rectum, 35% of the UC patients show the disease extending beyond the sigmoid (left-sided colitis) but no involvement of the entire colon is seen, and the rest 20% of the patients has the ulceration in the whole colon which is termed pancolitis. (150). The ulceration is most severe in the distal region of the colon than in the proximal part. The ulceration is continuous and uniform which is the hallmark of UC, with an exact transition between the affected and non affected regions. The macroscopic appearance of mucosa of UC during initial stages are hyperemic, edematous and granular. During the progression of the disease, the mucosa becomes haemorrhagic with visible punctuate ulcers. Ultimately the ulcers develop till the lamina propria with irregular shape with overhanging edges. As a result of recurrent episodes epithelial regeneration will take place along with the formation of pseudopolyps, which are the characteristic feature for the acute and chronic disease states. Atrophic colonic mucosa is associated with loss of colonic mucosal folds or haustra and with shortening and narrowing of the colon. Patients who are affected severely with this disease developed acute dilatation of the colon, with thin bowel wall and total ulceration seen in the entire mucosa with very less non-inflamed fragments of the colon. Microscopically UC in its acute stage is characterized by the edema of the lamina propria, congestion of capillaries and venules, with extravasation of erythrocytes which is followed by acute inflammatory cell infiltrate of neutrophils, lymphocytes, plasma cells, and macrophages which are accompanied by up-regulation of eosinophils and mast cells. Cryptitis and crypt abscesses will be seen due to neutrophilic infiltration of colonic crypts which is associated with discharge of mucus from

goblet cells and increased epithelial cell turnover and neutrophilic accumulations in crypt lumens. Even though all these changes were seen during the onset of the disease none of these above histologic features are specific for UC. (151,152).

Clinical features:

Patients with UC have different kinds of symptoms. Common symptoms include diarrhea, rectal bleeding, passage of mucus, tenesmus, urgency, and abdominal pain. Fever and weight loss may be significant during disease severity. These symptoms vary depending on the severity of the disease condition(153). The onset of the disease can be due to the exposure of the host to infectious microorganisms like *Clostridium difficile* or cytomegalovirus. Symptoms of UC will usually be present for weeks to months and the median interval between the onset of the disease and the diagnosis is approximately 9 months (154). It is always a query whether *Salmonella* or *C. difficile* are the initiating factors when the patient is initially diagnosed for normal colitis due to infectious agents and develops UC after a specific period of time. Rectal bleeding is common in UC and it solely depends on the widespread inflammation in the colon. Proctitis patients also show discharge of fresh blood either separately or streaked with the surface of the normal or hard stool. When the disease spreads throughout the colon there will be gross blood seen or blood is mixed with stools. During severe disease, patients pass liquid stool containing blood, pus and fecal matter. Active UC is confirmed with macroscopically evident blood . Diarrhea is common but not always seen. It may not be seen in 5% of patients with UC who may have proctitis with constipation and hard stools. The severity of the disease is indicated in patients with complain of passing loose stools or liquid stools with nocturnal diarrhea (155). Different mechanisms are involved in the pathophysiology of diarrhea in UC patients but failure to absorb salt and water is the major factor which resulted in Na/K-ATPase activity, increased mucosal permeability, and

altered membrane phospholipids. Lipid inflammatory mediators seems to be high in mucosal concentrations of UC patients which are seen even in normal colon which exerts chloride secretion and so there is a possibility for these mediators to cause diarrhea in UC patients by increasing mucosal permeability(156,157). Abdominal pain is one of the symptoms with severe active UC. Recurrent attacks results in severe cramping and abdominal pain but the cause of the pain is unknown and may be due to increased tension within the inflamed colonic wall during muscular contraction. Other symptoms include anorexia and nausea and during severe attacks actual vomiting will be seen. Weight loss occurs due to protein loss, hypercatabolism, and down-regulation of albumin synthesis. (158).

Laboratory findings:

Laboratory abnormalities can be seen in patients with severe UC or recurrent attacks. Hematologic changes include anemia, leukocytosis and thrombocytosis. Biochemical abnormalities include hypokalemia, metabolic alkalosis,and elevated serum levels of blood urea nitrogen and creatinine may be present in flares of UC cases. Erythrocyte sedimentation rate, C reactive protein are the serum inflammatory markers elevated during the active phase of the disease. Higher levels of these markers are neither sensitive nor specific for UC but measuring them gives the disease activity of the individual patients because these values usually will be normal during initial phase of the disease. CRP is more sensitive test when compared to ESR to follow-up the patients for the assessment of clinical changes because of the shorter half-life of CRP.(159).

Diagnosis:

At present, there is no single test available for the diagnosis of UC. So diagnosis can be made on endoscopic appearances and histologic findings. Stool cultures can be done in order to exclude infection with routine bacterial infections. Assay for toxins A, B and *C. difficile*, examining for ova and parasites can also be performed. (159).

Differential diagnosis

This includes infections of the colon such as amebiasis, shigellosis, cytomegalovirus infection, herpes simplex infection, enterohAemorrhagic *E. coli* infection, *C. difficile* infection and *Campylobacter* infection. Non-infectious causes include Crohn's disease, radiation colitis, ischemic colitis and solitary rectal ulcer. (159).

Assessment of disease activity

Several classifications are available to describe the severity of ulcerative colitis. These include the Truelove-Witts classification, UC disease activity index (UCDAI) and Mayo index. These are shown in the following Tables.

Truelove and Witts Classification

Mild
<4 stools/day, without or with only small amounts of blood No fever No tachycardia Mild anemia Erythrocyte sedimentation rate < 30 mm/hr
Moderate
Intermediate between mild and severe
Severe
>6 stools/day, with blood Fever > 37.5?C

Heart rate > 90 beats/min Anemia with hemoglobin level < 75% of normal Erythrocyte sedimentation rate > 30 mm/hr

Adapted from Truelove SC, Witts LJ. Cortisone in ulcerative colitis: Final report on a therapeutic trial. Br Med J 1955; 2:1041.

Ulcerative Colitis Disease Activity Index*

SCORE	CRITERIA
Stool Frequency	
0	Normal
1	1-2 stools/day > normal
2	3-4 stools/day > normal
3	>4 stools/day > normal
Rectal Bleeding	
0	None
1	Streaks of blood
2	Obvious blood
3	Mostly blood
Mucosal Appearance	
0	Normal
1	Mild friability
2	Moderate friability
3	Exudation, spontaneous bleeding
Physician Global Assessment	
0	Normal
1	Mild
2	Moderate
3	Severe

(From Sutherland LR, Martin F, Greer S, et al. 5-Aminosalicylic acid enema in the treatment of distal ulcerative colitis, proctosigmoiditis, and proctitis. Gastroenterology 1987; 92:1894.)

* Sutherland index: Range, 0-12.

Endoscopic and Histologic Assessment of Disease Activity in Ulcerative Colitis

SCORE	CRITERIA
Endoscopic Assessment	
0	Normal mucosa
1	Loss of vascular pattern

SCORE	CRITERIA
2	Granular, nonfriable mucosa
3	Friability on rubbing
4	Spontaneous bleeding, ulceration
Histologic Assessment	
0	Normal
1	No significant inflammation: Possibly architectural changes of chronic disease and small foci of lymphocytes but no acute inflammation, crypt abscesses, or epithelial destruction
2	Mild to moderate inflammation: Edema, vascularity, increased acute and chronic inflammatory cells but intact epithelium
3	Severe inflammation: Heavy infiltrate of acute and chronic inflammatory cells, crypt abscesses, ulceration of surface epithelium, purulent exudate

(Adapted from : Sleisenger, Fordtran, Gastrointestinal and Liver Disease, Pathophysiology, Diagnosis and Management 9th edition. Volume 2. Elsevier, 2010.

Baron JH, Connell AM, Lennard-Jones JE: Variation between observers in describing mucosal appearances in proctocolitis. BMJ 1964; 5375:89.

Truelove SC, Richards WC: Biopsy studies in ulcerative colitis. BMJ 1956; 4979:1315.

4. STEM CELLS IN INFLAMMATORY BOWEL DISEASES:

Stem cell alterations in IBD

Ulcerative colitis and Crohn's disease are characterized by mucosal ulceration (loss of the surface cells of the colon epithelium), crypt loss, and intestinal inflammation. Each of these changes is likely to lead to alterations in signaling that eventually lead to changes in stem cell number, stem cell location, and stem cell differentiation. These changes have not been systematically characterized in inflammatory bowel disease. Out of the four signaling events that are operative in the intestinal stem cells to regulate different functions in order to maintain intestinal homeostasis, Wnt signaling plays a major role on regulation of intestinal epithelial stem cell proliferation by their elegant mechanism of action to drive their receptors into nucleus for its function to take place. There are significant changes seen in stem cells during

inflammation in the colon which leads to colon cancer and defects in Paneth cell differentiation. Such differences can be observed in different Wnt receptors and ligands during normal and colitis condition. For example, Wnt 5A and Wnt 5B were highly expressed in normal small intestine and colon myofibroblasts and in UC and Crohn's patients myofibroblasts as well as in normal mouse colon. The different levels of expression between Wnt 5A and Wnt 5B was seen in both murine and human colon at the crypt base, such that Wnt 5B was more in murine colon whereas in human colon Wnt 5A was expressed in significant levels; in both cases, expression was at the base of the crypts. There are lower levels of expression of Wnt inhibitors such as sfrp1, sfrp4 and Dickkopf 1 observed in UC and Crohn's patients when compared with controls which represents the need for higher proliferation rate during epithelial repair upon injury(160,161). In chemical colitis which is induced by dextran sodium sulphate or trinitrobenzene sulfonic acid, the stem cells did not appear to have any significant role in repairing the epithelium even though the stem cell proliferation marker Musashi-1 was upregulated during injury and regeneration which is present at the 4^{th} tier of the basal region at the same time the specific lrg -5 marker was absent during repairing mechanisms since its presence is confined in the crypt based columnar cells. The mechanisms of initiating events and the progression of IBD varies in UC and Crohn's, but there is no difference in dysfunction seen in antigen presenting cells either in UC or CD, or hyperactivation of CD 4+ T cells(162). The cause for endothelial cell reduction is not known in IBD patients but the levels of VEGF increases which causes the procurement of endothelial cells in inflammatory bowel disease patients but experimental study shows decreased levels of these cells in IBD patients(163).

Stem cell therapy in IBD

Stem cell therapy has been mooted as a useful therapy to cure IBD. The object of because of Crohn's disease recurrence and mucosal impairment in Ulcerative colitis. Because synchronizing effects of intestinal stem cells can repair the damaged epithelial tissue and restore immunological abnormalities concurrently. Generally stem cells are indispensable to preserve the integrity of all adult tissues and due to its accuracy and regulation in differentiation, the adult tissues has the ability to regenerate the whole tissue and considered as a therapeutic agent for the degenerative diseases. Embryonic stem cells has the capability to differentiate into intestinal epithelial tissue and as immune cells due to its pluripotent nature it helps to cure colitis in murine model and paves the way to treat human colitis(164). Owing to regenerative, trophic and immunoregulatory functions of stem cells, hematopoietic and mesenchymal stem cells play a vital role in curing the disease. Various experimental models have proved that stem cell infusion can be used directly to patients due to its immunosuppressive effects(165).

Mesenchymal stem cells help in repairing of the inflamed intestine of UC patients whereas CD is characterized by fibrosis which repeatedly forms strictures and obstructions. On account of continuous presence of mesenchymal cells and hyperplasia, tissue disorganization and fibrillar collagen deposition, occurrence of excessive fibrous tissue became the major drawback for IBD patients. The biological difference between the normal intestinal fibroblasts and the IBD fibroblast is characterized by increased proliferation rates of cells and up-regulation of the secretion of collagen confirms the activity of the disease condition. Mesenchymal stem cells have the ability to differentiate into a variety of cells and help in modulation of immune response and repair inflamed epithelial tissue by secreting various cytokines and growth factors to the colitic area use the paracrine activity for its migration to the affected areas(166,167,168). Cells which have similarities in showing the mesenchymal stem cell properties can be isolated from

different tissues sources but with little difference in the yield and differentiation. The best population with good quality of MSCs are from the bone marrow source and these cells can be obtained by in vitro culture. Numerous studies have proved that MSCs are able to migrate to wound region and repair the tissue. Additionally MSCs suppress allogenic T cell proliferation and so they will not elicit an immune response after transplantation in immunocompetent recipients. Adipose derived MSCs have the great potential in healing perianal manifestations. Differentiated MSCs can be helpful in the tissue regeneration towards intestinal lineages(169).

Hematopoietic stem cells are multipotent cells which renew the entire hematopoietic system after the complete depletion of bone marrow cells (myeloablation). Since these cells are capable of forming endothelial precursors it will have the ability of intestinal tissue repair upon injury. It is well understood that impairment in the immune cell function causes the changes in mucosal barrier function which plays a vital role in the deregulated and prolonged inflammatory response in IBD. Even though a high dose of immune cells are removed, it can eliminate only the injurious T-lymphocytes but after hematopoietic stem cell transplantation hematopoiesis might occur and naïve cells will be produced that can restore tolerance. The HSC will create new immune system in the IBD patients. Further evidence suggests that allogenic transplantation of HSC and MSC have shown that both can repopulate the affected regions of the colon in experimental colitis in rat model which were induced by TNBS and improved gross morphologic scores(170). Further experimental observations suggests that topical implantation of labeled mesenchymal stem cells into TNBS induced chemical colitis by intravenous injections of bone marrow derived cells after characterizing with specific antibodies like CD29 and CD90 ameliorates colitis in rat models. The bone marrow cells were observed in the colonic wall of the intestine. The engrafted mesencymal stem cells were highly positive for the antibodies vimentin,

whereas expression of alpha-smooth muscle actin and desmin were less obvious(171). There were observations made for the bone marrow stem cell transplant in mouse models. Even if one transplanted bone marrow stem cell can proliferate the entire gut mucosa to 10-50 folds after a specific period of time, it is not clear whether these cells were short lived cells or long lived epithelial cells which were undifferentiated and multipotent throughout their plasticity. But the nature of intestinal stem cells that forms clonally derived units called crypts or stem cell clones suggests that these stem cells are not formed from bone marrow derived cells(172). Autologous bone marrow stem cell transplantation by intravenous injection from Crohn's disease patients recently explored that there is a minimal effect of these cells in endoscopic improvement in only 2 patients out of 10 tested, even though the bone marrow stem cells derived from Crohn's disease patients were similar to that of control subjects and characterization of these cells with antibodies by flow cytometry also supported this concept. The major disadvantage of this bone marrow or mesenchymal stem cell infusion by intravenous injection is that the number of cells homing to the inflammation site could not be determined and some of the cells would have been trapped into lungs (173). Some of the studies showed that mesenchymal stem cells derived from embryonic stem cells have the same effect in the remission of inflammatory bowel disease but at the same time mesenchymal stem cells obtained from induced pluripotent stem cells (iPS) showed a significant difference in the stem cell pattern due to the addition of ectopic factors during their conversion (174). Above all these, transplantation methods and direct delivery of mesenchymal stem cells into the inflammation site which indirectly acts to repair the epithelium (175). The intestinal subepithelial myofibroblast which is found beneath the basement membrane secretes various growth factors and cytokines through the paracrine mechanism, and has a very close link with the intestinal epithelium that would play a vital role in the mucosal repair.

Isolation of these cells might provide a direct stem cell therapy because of its genuine secretion of transforming growth factor β, epidermal growth factor, acidic and basic fibroblast growth factor (aFGF, bFGF) and proinflammatory cytokines (176).

5. STEM CELLS IN OTHER COLONIC DISEASES

It is widely accepted that tumour development is a multistep process which involves both mutations and epigenetic changes. Stem cells are the target for carcinogenesis due to their long life span and self renewing properties, and they have the capability of accumulating mutations when compared to short lived progenitors and more differentiated cells(177). The stem cell hypothesis states that tumours are originated from the cellular components which has stem cell properties, even though monoclonal in origin, most tumours shows cellular heterogeneity in their functional and morphological characteristics. According to this theory, a cancer cannot be created or expanded from a monoclonal expansion of a single cell but already transformed cell able to generate progeny of cancer cells that become heterogenous during differentiation. The major difference between the cells in tumors is assessed by its proliferative potential. The stem cell concept that tumours contain stem cell population with stem cell properties is first confirmed by clonogenic assays by culturing the cells which are isolated from tumor tissue and showed that only a small portion of the cells have a high proliferative capacity indicated by the various number of colonies produced in soft agar. Additionally more number of primary human cancer cells were injected into the immunocompromised mice in order to obtain tumour formation which explains that only a small number of cancer cells that reside in the tumour have the ability of tumorogenic potential. 5%-10% of the colon cancers are caused due to hereditary cancer syndromes(178,179). Mutations in tumour suppressors and oncogenes have the role in parallel progression of the disease along the adenoma –carcinoma sequence, and this led Fearon and

Vogelstein in 1990 to propose a model of successive genetic changes leads to colon cancer. These authors have suggested that mutation in KRAS and p53 which are responsible for controlling cell proliferation and genome integrity were important for tumour development(180,181). Colon cancer has been regarded as the disease of colonic epithelial stem cells. Recent evidence proved this hypothesis that colonic tumor arises from the stem cell origin. Colon cancer initiating cells were identified by two research groups in human tumours. Colon cancer initiating cells contain undifferentiated cells characterized by the expression of CD133 a cell surface marker, generally found on stem or progenitor cells of various tissues. CD133 positive cells were isolated from human colonic tumour which shows positivity of other markers such as BerEP4 (also known as ESA and Epcam) but not differentiation markers Cytokeratin 20 which is a filament protein present in the differentiated cells of the intestinal epithelium. CD 133 cells are also found in normal colonic tissues sparsely. This suggests that upregulation of CD133 positive cells in colon cancer cells arises from the oncogenic transformation of normal colonic stem cells. The subcutaneous injection of 3000 CD 133 positive cells isolated from fresh human tumour is capable of reproducing the original tumour and even very few like 100 CD 133 positive cells injected via subrenal capsule also reproduced similar human tumors. This *in vivo* limiting dilution experiments allowed the investigators to find out frequency of stem cells in colon tumours. These two experiments confirmed that approximately there is only one colon cancer initiating cell for every 5.7x10 unfractionated colon cancer cells and one CC-IC in every 262 CD 133 positive cells. CD 133 negative cells could not form any tumors(182,183,184.).

SCOPE AND PLAN OF WORK

SCOPE

There has been significant interest in stem cells in various tissues in recent years and there are important therapeutic applications relating to tissue and organ regeneration, amelioration of genetic defects and amelioration of haematological and immune defects. This interest has been spurred by the advancement of our knowledge of the basic biology of stem cells. While stem cells in certain tissues (eg. bone marrow) are extensively characterized, the study of stem cells in the gastrointestinal tract has lagged behind a little. In the last decade there has been considerable work on epithelial stem cells in the small intestine and their location, number, and signalling mechanisms have been elucidated. There is still little information available on epithelial stem cells in the colon. The colon is the site of several major diseases such as ulcerative colitis and Crohn's disease which are inflammatory diseases characterized by extensive ulceration and complications secondary to this. The scope of the studies described in this thesis was to determine the following:

(a) To isolate and to expand multipotent epithelial stem cells from the colon in order to be able to use it in possible therapy
(b) To identify alterations in epithelial stem cells in the colon in experimental colitis in animals
(c) To determine whether it will be possible to seed isolated proliferative epithelial cells from a donor animal into a recipient animal with severe experimentally-induced colitis; and
(d) To identify alterations in colonic epithelial stem cell number and distribution and signalling in human ulcerative colitis.

The institution in which these studies were carried out has a centre with the capacity to expand stem cells for clinical use in compliance with GMP requirements. Thus, any information generated in the project would be within the scope of *early product development*.

PLAN OF WORK

The plan of work was as follows:

(a) To isolate and to expand multipotent epithelial stem cells from the colon in order to be able to use it in possible therapy.

   a. The isolation would be done by divalent cation chelation using methods that have been in use in the guide's laboratory for two decades.
   b. Stem cell characteristics would be assessed by:
      i. Biological assays for stemness – clonogenic assay & growth in suspension culture
      ii. Expression of proteins associated with colonic epithelial stem cells by immunocytochemistry
      iii. Expression of mRNA of genes associated with colonic epithelial stem cells by reverse transcriptase PCR

(b) To identify alterations in epithelial stem cells in the colon in experimental colitis in animals

   a. Colitis would be induced in female adult wild-type Swiss albino mice by oral administration of dextran sodium sulphate
   b. Numbers and location of colonic epithelial stem cells would be ascertained by immunohistochemistry for cells expressing colonic epithelial stem cell markers.

(c) To determine whether it will be possible to seed isolated proliferative epithelial cells from a donor animal into a recipient animal with severe experimentally-induced colitis

   a. Animals would have a colonic infusion of donor epithelial cells from a male mouse donor at the height of the colitis.

b. Animals with colitis would be sacrificed and histological assessment made and Y chromosome tracking of the donor epithelial cells would be made (by fluorescence *in situ* hybridization).

(d) To identify alterations in colonic epithelial stem cell number and distribution and signalling in human ulcerative colitis.

a. Collect biopsies from patients with ulcerative colitis and controls with irritable bowel syndrome or rectal bleeding who are undergoing colonoscopy to rule out structural colonic disease.

b. Describe location and number of epithelial stem cells using immunohistochemistry for markers associated with colonic epithelial stem cells.

c. Identify changes in stem cell signalling in the colonic mucosa in ulcerative colitis by comparing mRNA expression of stem cell associated genes in colonic biopsies from patients with ulcerative colitis compared with controls without colonic mucosal disease.

METHODS

HUMAN PARTICIPANTS:

Human participants were recruited for these studies from those patients who were undergoing colonoscopy in the Department of Gastrointestinal Sciences of this hospital and were recruited into cases or controls depending on their satisfying inclusion criteria.

Inclusion criteria:

Ulcerative colitis:

1. Patients who were diagnosed to have ulcerative colitis, based on consensus Asian criteria that include clinical information, colonoscopy findings, biopsy findings consistent with idiopathic ulcerative colitis, and exclusion of infections (Ouyang et al, 2006). All patients seen in the Inflammatory Bowel Diseases Clinic and the Gastroenterology Clinic of this hospital underwent investigation according to a defined protocol that included colonoscopy and segmental ileo-colorectal biopsies. Colonoscopy was performed in the Endoscopy Unit and all biopsies were reported under the supervision of specialist gastrointestinal pathologists.
2. Age 18 years or older, of either sex.
3. Ability to give informed consent.

Control participants:

1. Patients with irritable bowel syndrome or rectal bleeding undergoing screening colonoscopy to exclude inflammatory bowel disease, colonic polyps or cancer.
2. Normal colonoscopy and rectal biopsy.
3. Age 18 years or older, of either sex.
4. Ability to give informed consent.

Participants were recruited in the Endoscopy Unit and written informed consent was obtained from all participants. The study protocol and the Patient Information and Consent forms were available in English and local language and they were approved by the Ethics Committee of the institution.

ANIMALS

Swiss albino inbred mice were used for these studies. The animals were locally bred in the Animal House of the Christian Medical College, Vellore, and were housed two to a cage and fed a commercial chow *ad libitum* and had free access to water provided in a bottle with dripper. Animal care and use protocols were followed as mandated by ethical guidelines for the use of animals. Animals were on average 3 months old and weighed approximately 25 g at the time of study. Male or female animals were used as necessary. Male animals were used as donors for experiments in which transplants were attempted, while female animals were recipients in these experiments. This allowed the tracking of male donor cells using fluorescence *in situ* hybridization for the Y chromosome (Y-FISH). For experiments in which animals were sacrificed, the animals were killed by administering an overdose of ether. For experiments in which the animals survived for varying periods of time, light ether anaesthesia was used to perform maneovures such as rectal instillation of enemas.

ISOLATION OF COLONIC EPITHELIAL SURFACE AND CRYPT CELL FRACTIONS AND VIABILITY TESTING

Epithelial cells were isolated from Swiss albino adult mice weighing around 25-30 grams of either gender by EDTA chelation technique. Mice were anaesthetized with ether, the colon was removed, flushed with water to eliminate the fecal contaminants, then twice with phosphate buffered saline (PBS) using a 5ml disposable plastic syringe. Subsequently the colon was everted over a small plastic tube, and the distal end was clamped. Calcium-free saline was injected into this everted colonic sac from the proximal end to achieve good distension of the colon, following which the proximal end was also clamped. The distended everted sac of colon was then placed in a 100 ml conical flask containing 50ml of PBS and 5mM EDTA, 1mM sodium butyrate (final concentration). Butyrate was added to the bathing solution as this is the major metabolic fuel for the colonocytes. The flask was placed in a shaking water bath (Tempe, USA) being continuously oxygenated. Every 10 minutes, the bathing solution was removed and the cells in the solution pelleted by gentle centrifugation at 800 rpm for one minute. The first fraction contained only surface cells. At the end of 45 minutes, the everted colon sac was removed, placed in 20 ml calcium-free PBS and shaken vigorously with a 1ml plastic pipette. The tight junctions were already loosened by the addition of EDTA and the physical manipulations allowed the crypt cells to be separated and isolated. The solution was transferred to a 50ml conical centrifuge tube, centrifuged at 800 rpm for a minute and the pellet was resuspended in 10ml of fresh PBS with 2mM DTT to remove the mucus (disulphide bonds from mucin). When necessary, cells were passed through a strainer to obtain a single cell suspension. One drop of the cell suspension was mixed with one drop of Trypan blue solution and this was placed in a Neubauer chamber. Cell viability was established by counting the number of cells as per usual protocol and cells in which

the nuclei stained with Trypan blue dye were considered non-viable, while cells that excluded Trypan blue were considered as viable.

Solutions needed for isolation of colonic crypts:

Phosphate-buffered saline (PBS). This was of the following composition:

NaCl	8 g
Na_2HPO4	1.16 g
KH_2PO_4	200 mg
KCl	200 mg
MilliQwater to	1 litre

This was autoclaved and labeled and was kept at 4°C for up to three weeks and then discarded.

EDTA 100mM stock solution: 37.22 mg of EDTA (Sigma Cat No. E5134, M.W.372.2) was dissolved in 100ml and autoclaved. This was stored at room temperature for up to 3 weeks for use and then discarded.

1mM sodium butyrate: PBS with EDTA was made as follows: To 50ml of PBS was added 2.5ml of EDTA from stock solution, which corresponds to 5mM final concentration of EDTA in PBS solution.

DTT (Sigma, Cat No D 0632 M.W. 154.2) always prepared in small quantity freshly, because it is unstable and with the time, temperature and pH it loses its reducing power (to reduce disulphide bonds). The final concentration was 2mM in 10ml PBS.

Cell viability Assay: (*Trypan blue exclusion test*)

0.4% W/V Trypan blue was prepared by dissolving 40mg Trypan blue in 10ml of MilliA water. This was stored at 4^0C in the refrigerator. Equal volumes were added to 50µl of cell suspension

(1:1), mixed with the pipette and placed a drop of this solution in the Neubaeur (haemocytometer) chamber for cell counting and viability assay. All four WBC ruling and RBC ruling areas were included for the cell count. The numbers of stained (non-viable) and unstained (viable) cells were counted, and the number of viable cells was expressed as a percentage of the total cells counted.

Chamber Indications	Upper right WBC	Upper left WBC	Lower right WBC	Lower left WBC	RBC	Total
Viable cells	110	70	74	67	80	80
Dead cells	0	3	10	7	15	7

EPITHELIAL CELL CULTURE:

The cells which were primarily used for culture are those located at the bottom of the crypts which are immature cells of the stem cell compartment and have the long term proliferative potential. The colon was removed and the epithelial cells were isolated in three different timed fractions at 15 minutes, 30 minutes and 45 minutes. These cells were resuspended in DMEM medium after counting and the assessment of viability with Trypan blue. The viable cells were seeded into each well after adding necessary culture conditions first to the wells in a 24 well collagen coated plate. The culture was done in triplicate in which the first horizontal four wells contained epidermal growth factor (EGF) and insulin. EGF and insulin-like growth factor receptors are present on epithelial cells and these factors are known to enhance the proliferation of epithelial cells in culture. In the second horizontal well culture supernatant of *Lactobacillus rhamnosus* strain GG was added. *Lactobacillus rhamnosus* strain GG is a well known probiotic. Lactobacilli are normal inhabitants of the colonic lumen. These bacteria secrete a number of

small molecules into the medium and we wished to check whether the bacterial supernatant would in any way alter proliferation of colonic epithelial cells. The third well contained conditioned medium obtained from HT-29 colon cancer cell line. The HT-29 cell line is an adenocarcinoma cell line that has colonic epithelial cell differentiation. Medium conditioned by these cells could potentially be rich in growth factors that help other epithelial cells grow and the inclusion of this "conditioned" medium was in order to determine whether it contained factors that would help in growth or replication of these primary epithelial cultures. The fourth well had the addition of supernatant of cultures of *Vibrio cholerae* which are cultured for 24 hours and their supernatant was taken. This organism is responsible for the disease cholera where there is severe acute watery diarrhoea. These bacteria secrete several toxins including cholera toxin. The latter is a potent stimulant of adenyl cyclase and increases the concentration of cyclic 3',5'-adenosine monophosphate. Cholera toxin has been used as a growth factor in epithelial cell culture, especially in primary colonic epithelial culture. The fifth well contained *Escherichia coli* culture supernatants. These bacteria are facultative anaerobes which are found in large numbers in the colon in healthy individuals. We considered that these bacteria might secrete factors into their culture medium that would influence epithelial cell growth. Finally the sixth well contained only DMEM medium without any of these additional factors and this was marked as control. 90% of DMEM was also added in all the wells along with 10% putative growth factor secreting supernatants.

Potential Growth Factors Tested	Concentrations added to Cells
EGF + Insulin	10ng/ml EGF, 0.25U/ml insulin
LGG culture supernatant	10% in DMEM medium
HT-29 conditioned medium	10% filtered culture supernatant

Filtered supernatants of the following bacteria grown to log phase under appropriate conditions.

Vibrio cholerae culture supernatant	10% in DMEM medium
E. coli culture supernatant	10% in DMEM medium
Control	DMEM alone .

Epidermal growth factor:

10ng/ml/well was the desired concentration, to achieve that we have taken 1µl from stock solution containing 1µg of EGF (Cat. No. 13247-051, Invitrogen) in 100µl sterile water and stored in Eppendorf tubes (cat no PCR-02-C, Axygen) at -20^0 C and have taken 1µl from this which contains 10ng. To avoid repeated freeze thawing, 10µl aliquots were stored in 10 different Eppendorf tubes.

Insulin (Human Insulin, Huminsulin $_{TM}$ 40 1U/ml).

Insulin was used instead of Insulin-like growth factor (IGF). We used 0.25units/ml/well, i.e. 6.25µl/well. All steps were carried out in a sterile condition. Insulin has previously been used to maintain colonic epithelial cells in primary culture.

Lactobacillus GG culture supernatant:

Lactobacillus GG (single colony from Rogosa SL agar plate) was incubated in 10ml MRS broth (Man Rogosa Sharpe broth, Hi Media, Cat. No. M 369) overnight at 37^0 C under anaerobic conditions in anaerobic jars in static culture. The desired quantity of broth was centrifuged at 1500 rpm using 15 ml conical centrifuge tube (Tarsons, Cat No. 546010) for 10 minutes and then filtered using Millipore syringe driven filter units (Cat no. SLGV 013 SL, 0.22µM). The culture filtrate was added to the DMEM culture medium at 10% concentration per well. 100 µl

conditioned medium + 900 µl DMEM medium were used per well. The culture supernate-filtrate was kept at -20^0 C for up to two weeks prior to use.

HT-29 conditioned medium:

HT-29 adenocarcinoma cell lines (obtained from National Centre for Cell Sciences, Pune) were cultured in our laboratory in DMEM medium along with 10% fetal bovine serum until they reached confluence. Usual laboratory protocols for maintenance of these cells was followed, and cells were split and subcultured when they reached confluence. The spent medium was changed every three days in sterile conditions. We have taken the spent medium after two passages, and this was first centrifuged at 1000 rpm for 10 minutes using IEC centrifuge and the cells which were sedimented down were discarded and the supernatant was filter sterilized by passage through Millipore syringe driven filter units (Cat no. SLGV 013 SL, 0.22µM) to avoid cell debris. From this, 10% conditioned medium was used for the primary culture of mouse colonocytes. 100µl conditioned medium + 900µl DMEM medium used per well. This conditioned medium was stored at -20^0 C for up to two weeks prior to use.

Vibrio cholerae culture supernatant:

A clinical isolate of *Vibrio cholerae* was cultured in 10 ml LB broth overnight in static culture at 37^0 C. The desired quantity of broth was centrifuged at 1500 rpm using 15 ml conical centrifuge tube (Tarsons, Cat No. 546010) for 10 minutes and then filtered using Millipore syringe driven filter units (Cat no. SLGV 013 SL, 0.22µM). 10% culture supernate was used per well. 100 µl conditioned medium + 900 µl DMEM medium used per well. This conditioned medium was stored at -20^0 C for up to two weeks prior to use.

Escherichia coli culture supernatant:

E. coli was cultured in a 10ml of nutrient broth overnight in an anaerobic jar at 37^0 C incubator in a test tube. The supernatant was removed in a 15ml conical centrifuge tube and was centrifuged at 1500 rpm using 15 ml conical centrifuge tube (Tarsons, Cat No. 546010) for 10 minutes and then filtered using Millipore syringe driven filter units (Cat no. SLGV 013 SL, 0.22µM). 10% culture medium was used per well. 100 µl conditioned medium + 900 µl DMEM medium used per well. This conditioned medium was stored at -20^0 C for up to two weeks prior to use.

Dulbecco's modified Eagle's medium:

This medium was prepared by readily available powder which was obtained from GIBCO BRL (Invitrogen , Chennai). The contents were unpacked and placed in 1 litre glass bottle and 1 litre of autoclaved Milli-Q water was added. The pH was checked which comes around 7.4 using the pH strips, and 7.5% of sodium bicarbonate was added to the medium to get the desired pH. Then it was mixed well with hand shaking. Then the medium was filter sterilized using a 500 ml filter unit with vacuum tube attached and stored as 500 ml aliquots at 4^0 C for up to one month before use. The antibiotics penicillin, streptomycin and amphotericin B were added as per the recommended concentrations.

Isolated colonocytes were cultured under different culture conditions to find out the suitable culture condition which aids in proliferation for up to 14 days. The colonocytes were cultured upto 14 days in collagen coated 24 well plates (Sigma, Z 380490, USA) with the above said conditions with change of medium for every 3 days. Cells were monitored using inverted microscope every day once to check any monolayer is formed in any of the culture conditions. At the end of 14 days cells were lysed using Trizol reagent (T9424, Sigma, USA) and RNA

concentrations were measured using fluorescence markers (Qubit, Invitrogen, Q32855, USA). RNA was converted to cDNA using MMLV reverse transcriptase. Quantitation of mRNA for the proliferation marker Musashi-1, and the regeneration marker Hes-1 was done by real time PCR using SYBR green in a chromo 4 (Bio rad) instrument.

CLONOGENIC ASSAY

Clonogenic assay measures the number and long-term proliferative potential of clonogenic cells within the epithelium. This assay can be used for the quantification of regeneration potential of the tissue *ex-vivo*. Similar assays are well established for the progenitor cells of tissues like bone marrow and to some extent for the epidermis. As far as the intestinal epithelium is concerned, limited success is available.

Materials needed for assay:

1. AGAR 1%. Agar Powder (Catalogue number:RM026) was obtained from Hi-Media Laboratories Pvt.Limited, Mumbai. 1 g of agar was dissolved in 100ml of MilliQ water and the whole agar mixture was autoclaved for 15 minutes. The autoclaved agar solution looked golden yellow in colour. Agar is generally prepared the previous day of the experiment and stored at 4^0 C and can be used for up to a week.

2. AGAROSE 0.8%. Agarose type VII A (Catalogue no. A0701) having low electroendosmosis (EEO) characteristics was obtained from Sigma, Bangalore. 0.8 grams was dissolved in 100ml of ultra pure water and autoclaved for 15 minutes resulting in a clear transparent solution. This was prepared on the previous day of the experiment and stored at 4^0 C for a week.

3. RPMI 1640 medium (2X) was obtained from GIBCO BRL (Invitrogen, Chennai). 10% FBS was added to the required quantity of medium.

Crypt disaggregation:

Colonocytes from mouse were isolated by the above said method and crypts were centrifuged and the cell pellet was resuspended in 20ml of 0.25% pancreatin (grade II, catalogue no. P1500; Sigma Chemical Co., St. Louis, MO) in PBS and incubated for 30 minutes at room temperature with occasional shaking. At the end of incubation period, the cell suspension was diluted with an equal volume of PBS and transferred to centrifuge tubes. After centrifugation at 1000 rpm for 5 minutes the supernatant fluid was discarded, and the cell pellet was resuspended in the EDTA/DTT mixture used to isolate the crypts. The tubes were centrifuged and the pellet resuspended in PBS. The cell suspension was checked by placing a drop on a microscope slide. If aggregates of cells are present, the cell suspension was gently drawn up into a syringe through a 21-guage needle 3 or 4 times, and the cell suspension checked microscopically. Occasionally the procedure needed to be repeated using a 23-guage needle. All of these manipulations were done gently because of the danger of disrupting the goblet cells in the cell suspension. The release of mucin causes the cells to aggregate. The cell suspension was checked microscopically and counted using a hemocytometer. The percentage of viable cells was again determined using trypan blue. If there were any small aggregates of cells, the cell suspension was filtered through a small cotton wool plug. The cell count was then repeated.

Clonogenic assay:

The above cell suspension was diluted if necessary in equal volumes of 0.8% agarose and 2X RPMI to produce a cell concentration of 50,000 cells /ml. Agar plates were prepared on the day of clonogenic assay. 2ml was aliquoted of the final suspension into the wells of the agar-coated plates. Then cooled the plates at 4^0C for 5 minutes to set the agarose and then placed the plates in

a sealed container holding a small volume of water to humidify the atmosphere and incubated at 37^0C for 3-4 weeks.

SUSPENSION CULTURE:

Suspension culture was carried out in isolated mouse colonocytes using Poly Hema (Poly 2-hydroxyethyl methacrylate, Sigma, P3932) coated plates. 2X RPMI 1640 medium was dispensed into all wells in a 6 well culture plate. Poly hema factor was prepared by dissolving in 95% ethanol and kept at 65° C overnight in an oven. This formed a viscous solution and was again diluted (1:10) with 95% ethanol and 1ml of this suspension was underlayed in each well, dried the 6 well plate after sealing with cover (in order to avoid contamination) overnight at 37^0 C This could be kept outside for long term storage. On the day of the culture, colonocytes which were isolated from the mouse colon by EDTA chelating technique were resuspended in equal volumes of RPMI 2X medium along with agarose type VII A and plated in the wells. The above cell suspension was diluted if necessary in equal volumes of 0.8% agarose and 2X RPMI to produce a cell concentration of 50,000 cells /ml. Cells after plating were again sealed tightly and kept at 37^0C in a 5% CO_2 incubator and inspected periodically for the formation of clones. Cells were monitored once in 3 days for the clones. The clones were formed on day 3, and on day 7 most of the single cells transformed into clones.

Preparation of Poly-Hema Stock & working solution:

0.6 grams of Poly-Hema powder was weighed and transferred to 8ml Laxbro vials and dissolved in 5ml of 95% ethanol. This preparation took more time to dissolve Poly-Hema powder so after the addition of 95% ethanol, they were kept overnight in hybridization oven at 65^0C for it to get dissolved properly. It formed a viscous solution the next day. The working solution was prepared as follows: Diluted 1:10 i.e. 1ml of Poly-Hema stock solution and 9ml of 95% ethyl

alcohol. 500µl of of this working solution was pipetted out and added to each well in a 6 well plate. Again the plates were dried overnight at 37^0C in an incubator after wrapping with aluminium foil.

AGAROSE 0.8%. Agarose type VII A (Catalogue no. A0701) having low electroendosmosis (EEO) characteristics was obtained from Sigma, Bangalore. 0.8 grams was dissolved in 100ml of ultra pure water and autoclaved for 15 minutes resulting in a clear transparent solution. This was prepared on the previous day of the experiment and stored at 4^0 C for up to a week.

RPMI 1640 medium (2X) was obtained from GIBCO BRL (Invitrogen, Chennai). 10% FBS was added to required quantity of medium.

At the end of 14 days, the 6 well plate (BD, Falcon 353046) was taken out from the incubator and monitored for clones which were in suspension. First all of the clones were pipetted out and transferred to a 15 ml conical centrifuge tube (BD, Falcon, Cat No. 352096). Some of the clones remained in the culture plates itself when viewed under the microscope when checked whether all the clones were removed, those clones were scraped by the pipette tip gently and have taken till the plate gets emptied along with Poly-Hema factor but there was no interference in RNA isolation because of Poly-Hema factor adhering to the plate. The clones were centrifuged at 1000 rpm without any breaks at 37^0 C, then the Poly-Hema factor was removed slowly and the clones were resuspended with PBS to wash the clones and some of the factor was still remained even after washing, and centrifuged again. The PBS was removed and 1ml of Trizol was added to the clones.

RNA was extracted from the clones using TRIzol reagent, converted to cDNA, and PCR for Musashi-1 and Hes-1 were done as described above.

IMMUNOCYTOCHEMISTRY

Colonocytes were isolated by EDTA chelation and single cell suspension made after isolation. This was used to make a smear on poly-l-lysine coated slides and air dried for 5 minutes and stored at -20° C until use. The cell smear was washed in PBS once for 5 minutes, fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 2% Triton X-100 (T8532, Sigma, USA) for one and half an hour. Then the cell smear was blocked with 3% bovine serum albumin (A2153, Sigma) in PBS for one hour, and incubated with primary antibody Musashi-1 (AB 5977, Chemicon) for three hours at room temperature. The cells were washed three times in blocking buffer (3% BSA in PBS) before secondary antibody application. Then fluorescence (fluorescein isothiocyanate, FITC) tagged anti rabbit antibody which was diluted in 3% (BSA) and applied to cells and incubated at room temperature for one hour. After three washes in PBS, DAPI (4,6 Diamidino-2– phenylindole, dihydrochloride D 1306) was applied and visualized under a fluorescence microscope.

IMMUNOHISTOCHEMISTRY (MUSASHI-1, HES-1)

Mouse colon was removed after anaesthetizing the animal with ether overdose and the lumen was cleansed with phosphate buffered saline by inserting 5ml syringe which was filled with saline, proximal and distal colon was separated and fixed in 4% paraformaldehyde immediately in ice and kept at 4^0C overnight to maintain the tissue integrity so that the antigenicity would not be lost and the sensitivity of the antibody could be maintained to find out the exact location of the antibody. The fixed colon was processed in graded alcohol overnight and in xylene for one and half hours in order to remove alcohol in a histokine machine. Next day the tissue was taken out using a forceps and embedded in paraffin wax in a wooden square block or plastic cassettes and sections cut to required thickness in a microtome.

Preparation of 4% paraformaldehyde for tissue fixation:

2 grams of paraformaldehyde (Sigma) was weighed and dissolved in 50ml of phosphate buffered saline in a 50ml glass conical flask and covered with parafilm. It was heated in a hot plate and brought it to boiling to dissolve completely. The solution was cooled for sometime at room temperature and stored at 4^0C for up to one week.

Preparing paraffin wax section for immunostaining:

Sections were cut at 4 μm thickness and labeled with pencil and placed on poly-L-lysine coated slides. The slides were kept at 37^0C overnight for the adherence of sections properly and not to get damaged when doing heat mediated antigen retrieval step. Commercially available poly-L-lysine coated slides were used for this procedure.

Localisation of Musashi-1 in mouse colon (normal and DSS colitis)

Mouse colon was fixed separately in 4% paraformaldehyde overnight at 4^0 C, and embedded in paraffin. Sections were cut and stained for the proliferative marker Musashi-1 (Chemicon, 5977). Tissue sections were dewaxed in xylene in two turns of 15 minutes each, rehydrated in the series of graded alcohol for not more than 1 minute except for absolute alcohol for 15 minutes. Washed the slides once with distilled water and PBS. Then methanol quenching was done in order to avoid endogenous peroxidase activity in the tissue sections which might interfere with false positive staining. After three washes in PBS, antigen retrieval was performed with heat mediated treatment in 10mM citrate buffer at pH 6.0 in a microwave for 15 minutes in high power. Then it was cooled in the same buffer in the running water bath for 15 minutes. Washed the slides first in tap water and then with distilled water and finally with 3 turns of PBS 5 minutes each. Blocking with 3% bovine serum albumin was done in order to avoid the non specific staining of the antibody, the mechanism behind this is all the epitope will be blocked during this step but if the

antibody is highly sensitive to the particular epitope it will bind to the antigenic site firmly. Then slides were tilted slightly to remove excess bovine serum albumin and wiped carefully along the sides of the tissue sections with tissue paper and slides were incubated with primary antibody Musashi 1 (5977 Chemicon, UK) diluted in PBS and Triton-x 100 (0.01%) to enhance the penetration of the antibody into the tissues and kept it in a humidified chamber carefully and incubated overnight at 4^0 C. The following day slides were taken out carefully and checked for the presence of antibody with the naked eye to confirm that no antibody solution was spilled or moved away from the tissue section due to capillary action in the slides, washed with PBS thrice at 5 minutes interval and Anti–rabbit secondary antibody (Catalog number, A0545, Sigma, US) conjugated with peroxidase was used as secondary antibody which was diluted in BSA blocking solution and incubated for one hour at room temperature and washed with PBS for three times and Di amino Benzidine (DAB, Sigma, US) was added and incubated for half an hour in dark and for the color detection for the positive staining. Then the slides were washed immediately in tap water to arrest the DAB reaction followed by 95% ethanol and 100% ethanol and cleared in xylene to remove any dust particles stuck to the slide and air dried for sometime till the ethanol gets completely removed and mounted with a glass cover slip and sealed with DPX mountant and visualized under the light microscope.

Localisation of HES-1 in mouse colon: (normal and DSS colitis)

Mouse colon was fixed separately in 4% paraformaldehyde overnight at 4^0 C, and embedded in paraffin. Sections were cut and stained for the proliferative marker HES-1 (Chemicon, 5702). Tissue sections were dewaxed in xylene in two turns of 15 minutes each , rehydrated in the series of graded alcohol for not more than 1 minute except for absolute alcohol for 15 minutes. Washed the slides once with distilled water and PBS. Then methanol quenching was done using

methanol quenching buffer which was prepared in PBS in order to avoid endogenous peroxidase activity in the tissue sections which might interfere with false positive staining. After three washes in PBS antigen retrieval was performed with heat-mediated treatment in 10mM citrate buffer at pH 6.0 in a microwave for 15 minutes in high power. Then it was cooled in the same buffer in the running water bath for 15 minutes. Washed the slides first in tap water and then with distilled water and finally with 3 turns of PBS 5 minutes each. Blocking with 3% Bovine Serum albumin was done in order to avoid the non specific staining of the antibody, the mechanism behind this is all the epitope will be blocked during this step but if the antibody is highly sensitive to the particular epitope it will bind to the antigenic site firmly. After one hour the slides were tilted slightly to remove excess bovine serum albumin and wiped carefully along the sides of the tissue sections with tissue paper and slides were incubated with primary antibody HES-1 (AB5702, Chemicon, UK) diluted in PBS and Triton-x 100 (0.01%) to enhance the penetration of the antibody into the tissues and kept it in a humidified chamber carefully and incubated overnight at 4^0 C. The following day slides were taken out carefully and checked for the presence of antibody with the naked eye to confirm that no antibody solution was spilled or moved away from the tissue section due to capillary action in the slides and kept it in room temperature for one hour for the fastest reaction between the antigen-antibody binding to take place and washed with PBS thrice at 5 minutes interval and Anti–rabbit secondary antibody conjugated with peroxidase was used as secondary antibody which was diluted in BSA blocking solution and incubated for one hour at room temperature and washed with PBS for three times and Di amino Benzidine (DAB) was added and incubated for half an hour in dark and for the color detection for the positive staining. Then the slides were washed immediately in tap water to arrest the DAB reaction followed by 95% ethanol and 100% ethanol and cleared in xylene to

remove any dust particles stuck to the slide and air dried for sometime till the ethanol gets completely removed and mounted with a glass cover slip and sealed with DPX mountant and visualized under the light microscope.

Localisation of Musashi -1 in normal and colitis human colon biopsy:

Colonic biopsies were obtained during colonoscopy of patients with ulcerative colitis and from appropriate controls undergoing screening colonoscopy. The biopsies were fixed separately in 4% paraformaldehyde overnight at 4^0 C, and embedded in paraffin. Sections were cut and stained for the proliferative marker Musashi-1 (Chemicon, 5977). Tissue sections were dewaxed in xylene in two turns of 15 minutes each , rehydrated in the series of graded alcohol for not more than 1 minute except for absolute alcohol for 15 minutes. Washed the slides once with distilled water and PBS. Then methanol quenching was done in order to avoid endogenous peroxidase activity in the tissue sections which might interfere with false positive staining. After three washes in PBS antigen retrieval was performed with Heat mediated treatment in 10mM citrate buffer at pH 6.0 in a microwave for 15 minutes in high power. Then it was cooled in the same buffer in the running water bath for 15 minutes. Washed the slides first in tap water and then with distilled water and finally with 3 turns of PBS 5 minutes each. Blocking with 3% Bovine Serum albumin was done in order to avoid the non specific staining of the antibody, the mechanism behind this is all the epitope will be blocked during this step but if the antibody is highly sensitive to the particular epitope it will bind to the antigenic site firmly. Then slides were tilted slightly to remove excess bovine serum albumin and wiped carefully along the sides of the tissue sections with tissue paper and slides were incubated with primary antibody Musashi 1 diluted in PBS and Triton-x 100 (0.01%) to enhance the penetration of the antibody into the tissues and kept it in a humidified chamber carefully and incubated overnight at 4^0 C. The

following day slides were taken out carefully and checked for the presence of antibody with the naked eye to confirm that no antibody solution was spilled or moved away from the tissue section due to capillary action in the slides, washed with PBS thrice at 5 minutes interval and anti–rabbit secondary antibody conjugated with peroxidase was used as secondary antibody which was diluted in BSA blocking solution and incubated for one hour at room temperature and washed with PBS for three times and Di amino Benzidine (DAB) was added and incubated for half an hour in dark and for the color detection for the positive staining. Then the slides were washed immediately in tap water to arrest the DAB reaction followed by 95% ethanol and 100% ethanol and cleared in xylene to remove any dust particles stuck to the slide and air dried for sometime till the ethanol gets completely removed and mounted with a glass cover slip and sealed with DPX mountant and visualized under the light microscope.

Localisation of HES-1 in Human Normal and Colitis Biopsy Tissue:

Colonic mucosal biopsies were fixed separately in 4% paraformaldehyde overnight at 4^0 C, and embedded in paraffin. Sections were cut and stained for the proliferative marker Musashi-1 (Chemicon, 5977). Tissue sections were dewaxed in xylene in two turns of 15 minutes each, rehydrated in the series of graded alcohol for not more than 1 minute except for absolute alcohol for 15 minutes. Washed the slides once with distilled water and PBS. Then methanol quenching was done using methanol quenching buffer which was prepared in PBS in order to avoid endogenous peroxidase activity in the tissue sections which might interfere with false positive staining. After three washes in PBS antigen retrieval was performed with heat mediated treatment in 10mM citrate buffer at pH 6.0 in a microwave for 15 minutes in high power. Then it was cooled in the same buffer in the running water bath for 15 minutes. Washed the slides first in tap water and then with distilled water and finally with 3 turns of PBS 5 minutes each. Blocking

with 3% bovine serum albumin was done in order to avoid the non specific staining of the antibody, the mechanism behind this is all the epitope will be blocked during this step but if the antibody is highly sensitive to the particular epitope it will bind to the antigenic site firmly. Then slides were tilted slightly to remove excess bovine serum albumin and wiped carefully along the sides of the tissue sections with tissue paper and slides were incubated with primary antibody HES-1 diluted in PBS and Triton-X 100 (0.01%) to enhance the penetration of the antibody into the tissues and kept it in a humidified chamber carefully and incubated overnight at 4^0 C. The following day slides were taken out carefully and checked for the presence of antibody with the naked eye to confirm that no antibody solution was spilled or moved away from the tissue section due to capillary action in the slides, and kept it in room temperature for one hour for the fastest reaction between the antigen-antibody binding to take place and washed with PBS thrice at 5 minutes interval and anti–rabbit secondary antibody conjugated with peroxidase was used as secondary antibody which was diluted in BSA blocking solution and incubated for one hour at room temperature and washed with PBS for three times and Di amino Benzidine (DAB) was added and incubated for half an hour in dark and for the color detection for the positive staining. Then the slides were washed immediately in tap water to arrest the DAB reaction followed by 95% ethanol and 100% ethanol and cleared in xylene to remove any dust particles stuck to the slide and air dried for sometime till the ethanol gets completely removed and mounted with a glass cover slip and sealed with DPX mountant and visualized under the light microscope.

FLUORESCENCE *IN SITU* HYBRIDIZATION (Y-FISH)

Colitis was induced in female mice weighing around 20-25 grams using dextran sulphate sodium according to the standard experimental protocol. Colitis was confirmed by Hemospot test card (Corel Clinical Systems, Goa, India) by measuring fecal occult blood from 3rd to 9th day. After severe colitis has been confirmed on 9th day, colonocytes from male mice was isolated and instilled into the rectum on day 10 through the anus to the female mice using a catheter and the localization of the colonocytes of the male mice was confirmed by tracking Y chromosome through fluorescence *in situ* hybridization.

RNA EXTRACTION

RNA was extracted from colonocytes using TRIzol reagent (Sigma, T9424,USA) following the manufacturer's instructions. At the end of 14 days, the culture dish was taken out from the incubator and the medium was removed. Phosphate buffered saline was added to all the wells in order to wash the cells once before RNA isolation. 1ml of Trizol reagent was added to all the wells and by using the cell scraper the cells were removed and taken in a separate 1ml vial and colonocytes were lysed using one ml of Trizol reagent by repetitive pipetting. Then 200µl of chloroform was added to 1ml of Trizol and mixed vigorously for 15 seconds followed by incubation in room temperature for 2-3 minutes. The mixture was centrifuged in a cooling centrifuge for 15 minutes. Three layers were formed after centrifugation in which the first layer, which is aqueous, contains the RNA. This aqueous layer was transferred to a new sterile Eppendorf tube. Then 500µl of isopropanol was added and incubated for one hour at -20^0 C to get a better yield. After one hour the tubes were centrifuged at 12,000 g at 4^0 C in a refrigerated centrifuge (Cat no PCR-02-C, Axygen) for 10 minutes. Then the supernatant was aspirated with micropipette and 1ml of 75% ethanol was added and mixed it using vortex. Then it was

centrifuged at 7500g for 5 minutes at 4^0 C and the ethanol was carefully removed using pipette and the RNA pellet was air dried for 5 minutes to remove the excess ethanol and water was added and dissolved using repeated pipetting and kept at 55-60^0 C in a water bath and stored at -80^0 C until use.

CONVERSION OF RNA TO cDNA:

The RNA was converted to single stranded DNA for studies involving measurement of mRNA expression by quantitative polymerase chain reaction. The first step was the annealing of random primers to the RNA then adding nucleotides and enzyme (reverse transcriptase) to extend the annealed primers into complementary DNA (cDNA).

PROTOCOL FOR cDNA CONVERSION:

RNA	1µg
Hexamer	4µl
Water	5µl

Spin the tubes in minicentrifuge

Keep the tubes in 70^0C for 5 minutes.

Immediately keep the tubes in ice.

Mix with Mastermix

Spin the tubes in microcentrifuge

Incubate in thermal cycler

Program 42^0C for 60 minutes

Thermal cycler conditions:

Initial Step	10 minutes 25^0C
Reverse Transcriptase Step	30 minutes 48^0C

Inactivation of the RT enzyme 5 minutes 95^0C

The RNA was converted to cDNA by using 10X reaction buffer, 25mM $MgCl_2$, 2.5mM dNTPs, random nanomers, RNAase inhibitors, Eurotranscript Reverse Transcriptase and RNAase free water and template (Total RNA) according to the manufacturer's instructions (Eurogentec, Reverse Transcriptase Core kit, Belgium).

POLYMERASE CHAIN REACTION AMPLIFICATION FOR MUSASHI-1 & HES-1:

PCR was carried out for Musashi-1 and Hes-1 which are considered as intracellular markers for intestinal stem cells. In cultured cells at the end of 14 days the cells were lysed using Trizol reagent and RNA was isolated and its concentrations were measured using fluorescence markers (Qubit, Invitrogen), and this is converted to cDNA using MMLV reverse transcriptase. Quantitation of mRNA for the proliferation marker Musashi-1, and the regeneration marker Hes-1 was done by real time PCR using SYBR green in a Chromo 4 (Bio Rad) instrument.

Primers for Mouse Musashi -1 and Mouse HES-1.

mMusashi-1

Forward: 5'- atggtggaatgcaagaaagc -3'

Reverse: 5'- taggtgtaaccaggggcaag -3'

mHes-1

Forward: 5'- tcatcaaagcctatcatggaga -3'

Reverse: 5'- cggaggtgcttcacagtcat -3'

PCR conditions for Musashi 1 and HES-1:

1. Initial denaturation 94◦ C for one minute.
2. Cycle denaturation 94◦ C for 30 seconds.

3. Annealing temperature 60.5◦ C for 30 seconds.

4. Extension 72◦ C for 30 seconds.

5. Total number of cycles = 45 cycles.

The same was followed for internal control GAPDH.

Gel electrophoresis

Materials required:

Agarose

Lithium Borate Buffer (5mM)

Lithium hydroxide – 4.19g

Boric acid – 20g

pH – 8.2

Fill to a final volume of 500ml with distilled water.

Gel-loading buffers:

0.25% (w/v) bromophenol blue

40% (w/v) sucrose

Ethidium bromide (0.5 μg/ml)

Agarose gel electrophoresis was used to separate and visualize DNA bands. A 2% gel made up with 5mM lithium borate buffer was cast. Gel loading buffer was added to the PCR product to obtain a final concentration of 1 X .The sample was mixed and loaded into the wells. 0.5 μg/ml of ethidium bromide was added to the buffer to visualize the amplicons. Electrophoresis was carried out at 150v for 40 minutes till the band separation is complete. A DNA marker running from 500bp to 50bp was used against which the PCR products are compared. The gel was visualized under the UV transilluminator (Vilbert Lourmat, France) to detect the bands.

PATHWAY-TARGETED STEM CELL PCR ARRAY FOR ANALYSIS OF GENE EXPRESSION

Tissue homogenization

Colonic biopsy samples from both ulcerative colitis patients and controls were collected and frozen in liquid nitrogen and transported to the laboratory and kept in -80^{0}C until use. On the day of RNA isolation, 1ml of Trizol was added to the sample and transferred to a 1ml glass vial and the sample was homogenized in a Teflon pestle tissue homogenizer for 5 minutes till the tissue was completely homogenized. Then it was transferred to a 1ml Eppendorf tube and RNA was isolated. The isolated RNA was purified using an RNA purification kit (Cat.No. 74104, Qiagen,USA). This kit is useful to cleanup RNA previously isolated by different methods. The protocol described here is first the isolated RNA sample was adjusted to a volume of 100µl with RNase-free water given along with the kit. 350 µl of the RLT buffer was added and mixed thoroughly with pipette. Then 250µl of ethanol was added to the diluted RNA, and mixed well by pipetting. The total volume is 700µl was transferred to an RNeasy Mini spin column placed in a 2ml collection tube which is supplied along with the kit and closed the lid gently, and centrifuged for 15 seconds at 10,000 rpm. The flow through was discarded from the collection tube. Then 500µl of RPE buffer was added to the RNeasy spin column closed the lid gently and centrifuged for 15 seconds at 10,000rpm to wash the spin column membrane. The flowthrough was discarded. 500µl RPE buffer was added to the RNeasy spin column and centrifuged for 2 minutes at 10,000rpm to wash the spin column membrane. RNeasy spin column was placed in a new 1.5ml collection tube provided in the kit and 30-50µl of RNase free water directly to the spin column membrane and centrifuged for a minute at 10,000rpm to elute the RNA.

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Two step cDNA conversion

This two step cDNA conversion was carried out using a kit commercially available along with a pathway-targeted PCR array. The RNA which was isolated and purified by the above said method was converted to cDNA by two step conversion method. In the first step, genomic DNA was eliminated by adding three components such as RNA, buffer GE, and RNase free water to make up the total volume up to 10 µl and placed the genomic DNA elimination mix for 5 minutes at 42^0C and placed immediately on ice for one minute. To this mixture, reverse transcription mix was added which contains buffer BC3, control P2, RE3, reverse transcriptase mix and the total volume was made up with 10µl RNase free water to 20 µl and mixed gently by pipetting up and down. This mixture was incubated at 42^0C for exactly 15 minutes and immediately stopped the reaction by incubating at 95^0C for 5 minutes. Then 91µl of RNase free water was added to each reaction and mixed up by pipetting up and down several times and stored at -20^0C until use for PCR array.

PCR array:

RNA was extracted from 2 mucosal biopsy bits, converted to cDNA as mentioned above. In order to determine the expression of genes related to growth, identification and differentiation of stem cells, the cDNA was then amplified in a stem cell gene pathway-focussed PCR array. PCR arrays are designed to profile the expression of pathway-specific genes. The Human Stem Cell Signaling RT² Profiler™ PCR Array (PAHS-047, SA Biosciences) was used for these studies. This array provides quantitative information on expression of genes related to receptors and transcription factors of the major signaling pathways involved in pluripotent cell maintenance and differentiation, including Fibroblast Growth Factor, Hedgehog, Notch, TGFβ and WNT. Monitoring the expression of receptors and co-receptors insures that stem cells can recognize the

necessary growth factors or other receptor ligands. The array is designed that mRNA expression of the target genes is amplified by real time PCR and we undertook the amplification in a Chromo 4 real time PCR instrument (Biorad, USA) using SYBR green.

The list of genes whose expression is detected in this array and the broad functional classes of these genes are listed below:

Table 4.1. Pathways included in analysis in the Stem Cell Signaling PCR Array.

Pathway	**Receptors**	**Transcription factors & Co-factors**
Pluripotency Maintenance	IL6ST (GP130), LIFR	STAT3
Fibroblast Growth Factor (FGF)	FGFR1, FGFR2, FGFR3, FGFR4	CDX2
Hedgehog	PTCH1, PTCHD2, SMO	GLI1, GLI2, GLI3, SUFU
Notch	NCSTN, NOTCH1, NOTCH2, NOTCH3, NOTCH4, PSENEN, PSEN1, PSEN2	RBPJL
TGFβ Superfamily	ACVRL1, ACVR1, ACVR1B, ACVR1C, ACVR2A, ACVR2B, AMHR2, BMPR1A, BMPR1B, BMPR2, ENG, LTBP1, LTBP2, LTBP3, LTBP4, RGMA, TGFBR1, TGFBR2, TGFBR3, TGFBRAP1	EP300, SMAD1, SMAD2, SMAD3, SMAD4, SMAD5, SMAD6, SMAD7, SMAD9, CREBBP, E2F5, RBL1, RBL2, SP1, ZEB2
Wnt	FZD1, FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, FZD9, LRP5, LRP6, VANGL2	BCL9, BCL9L, CTNNB1, LEF1, NFAT5, NFATC1, NFATC2, NFATC3, NFATC4, PYGO2, TCF7L1, TCF7L2, TCF7

Performing real-time PCR detection:

1. Sample Preparation:

Procedure for cocktail preparation for PCR array plate

a. Experimental Cocktail #1 for wells A1 through H5

The following components were mixed in a 5-ml tube or a multi-channel pipettor reservoir:

2X SuperArray PCR master mix	1225 µl
Diluted first strand cDNA synthesis reaction	98 µl
ddH2O	1127 µl
Total volume	2450 µl

b. Experimental Cocktail #2 for wells H6 through H12 for the internal controls and NTC or NRT: 100 µl of 2X PCR Master mix mixed with 100 µl of ddH2O to generate 1X PCR Master Mix. Then, following procedure was followed.

"Experimental Cocktail #2 for Controls"

In the first five wells of the strip, prepared five (5) 10-fold serial dilutions of Experimental Cocktail #1. 3 µl of Experimental Cocktail #1 was mixed with 27 µl of Experimental Cocktail #2 to generate four more 10-fold serial dilutions of this mixture in the same fashion.

For WELL	H6	H7	H8	H9	H10
	27 µl	27 µl	27 µl	27 µl	27 µl

Experimental Cocktail #2

Material used for previous tube	3 µl	3 µl	3 µl	3 µl	3 µl

In the sixth tube of the strip, 1 µl of a 1:100 dilution of original input total RNA was mixed with 24 µl of Experimental Cocktail #2 to use as the no reverse transcription (NRT) control in well H11.

In the seventh tube of the strip, 25-µl aliquot of Experimental Cocktail #2 was used as the no template control (NTC) in well H12.

Program: the three-step cycling program,

Cycles	Duration	Temperature
1	10 minutes 1	95 °C
40	15 seconds	95 °C
	30 to 40 seconds 2,3	55 °C
	30 seconds	72 °C

- The 10-minute step at 95 °C is required to activate the HotStart DNA polymerase.
- Detect and record SYBR Green fluorescence from every well during the annealing step of each cycle.
- Different instruments need different lengths of time to detect the fluorescent signal. The annealing step (55°C) time needs to be fixed as appropriate for each instrument.
- Calculated the threshold cycle (Ct) for each well using the instrument's software.

Quality Control 1: Dissociation (Melting) Curve:

Melting curve program was run immediately after the above PCR program, to generate a first derivative dissociation curve for each well in the entire plate using the instrument's software. No more than one peak appearing in each reaction at temperatures greater than 80°C was considered to avoid non-specific amplification. 95°C, 1 min; 65°C, 2 min (OPTICS OFF); 65°C to 95°C at 2°C / sec (OPTICS ON).

Quality Control 2: Agarose gel electrophoresis:

The other optional to test the quality control, agarose gel electrophoresis was done. Once the PCR was completed the plates were saved at -20°C to run a gel electrophoresis to check the non-specific amplification and for troubleshooting purposes. No more than one band should be apparent in each lane in specific amplification. 10 µl of each reaction was mixed with 2 µl of 6X agarose gel loading buffer and loaded each sample into separate wells of a 2% agarose gel containing 0.5 µg/ml ethidium bromide in 1X TBE with an appropriate amount of 100-bp DNA Step Ladder in an adjacent lane. Electrophoresis was run in 1X TBE at 90V for 40 minutes or before the tracking dye runs off the gel. Then image of the gel was documented in a ChemiSmart (Vilber-Lourmat, France).

Data Analysis: ΔΔ Ct Method:

Once the PCR was done the threshold cycle of the negative controls was examined. The No Template Control (NTC) tests and No Reverse Transcription (NRT) control tests were included in the PCR array to show the DNA contamination and the original RNA with genomic DNA. In both the threshold cycles greater that 35 are considered to be free of DNA contamination. If the threshold cycle for either of these controls is less than 35, then the presence of DNA contamination is evident. Ct values were reported as greater than 35 or as N/A (not detected) to 35, any Ct value equal to 35 was considered a negative call.

The ΔCt was calculated for each pathway-focused gene in each group.

ΔCt (group 1) = average Ct – average of HK genes' Ct for group 1 array

ΔCt (group 2) = average Ct – average of HK genes' Ct for group 2 array

The use of housekeeping (HK) genes is based on the principle that the expression level of the housekeeping genes chosen for normalization in the ΔΔCt method must not be influenced by the

experimental conditions. An appropriate housekeeping gene was used, the average Ct value of all five housekeeping genes, but only if the difference in the average values between the two groups to be compared is less than one (1) cycle the average of HK genes' Ct for each group to be compared, to rely on the consistency in the quantity and quality of the original input of total RNA across the groups to effectively normalize the results.

The ΔΔCt was calculated for each gene across groups.

ΔΔCt = ΔCt (group 2) - ΔCt (group 1)

Where group 1 is the control and group 2 is an experimental group.

The fold-change for each gene from group 1 to group 2 was calculated as 2^ (-ΔΔCt).

Table 4.2. List of genes whose expression is detected in the stem cell signalling PCR array.

Position	Unigene	Symbol	Description	Gname
A01	Hs.470316	ACVR1	Activin A receptor, type I	ACTRI/ACVR1A/ACVRLK2/ALK2/FOP/SKR1/TSRI
A02	Hs.438918	ACVR1B	Activin A receptor, type IB	ACTRIB/ACVRLK4/ALK4/SKR2
A03	Hs.562901	ACVR1C	Activin A receptor, type IC	ACVRLK7/ALK7
A04	Hs.470174	ACVR2A	Activin A receptor, type IIA	ACTRII/ACVR2
A05	Hs.174273	ACVR2B	Activin A receptor, type IIB	ACTRIIB/ActR-IIB/MGC116908
A06	Hs.591026	ACVRL1	Activin A receptor type II-like 1	ACVRLK1/ALK-1/ALK1/HHT/HHT2/ORW2/SKR3/TSR-I
A07	Hs.659889	AMHR2	Anti-Mullerian hormone receptor, type II	AMHR/MISR2/MISRII
A08	Hs.415209	BCL9	B-cell CLL/lymphoma 9	LGS/MGC131591
A09	Hs.414740	BCL9L	B-cell CLL/lymphoma 9-like	BCL9-2/DLNB11
A10	Hs.524477	BMPR1A	Bone morphogenetic protein receptor, type IA	10q23del/ACVRLK3/ALK3/CD292/SKR5
A11	Hs.598475	BMPR1B	Bone morphogenetic protein receptor, type IB	ALK-6/ALK6/CDw293
A12	Hs.471119	BMPR2	Bone morphogenetic protein receptor, type II (serine/threonine kinase)	BMPR-II/BMPR3/BMR2/BRK-3/FLJ41585/FLJ76945/PPH1/T-ALK
B01	Hs.174249	CDX2	Caudal type homeobox 2	CDX-3/CDX3
B02	Hs.459759	CREBBP	CREB binding protein	CBP/KAT3A/RSTS
B03	Hs.476018	CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	CTNNB/DKFZp686D02253/FLJ25606/FLJ37923
B04	Hs.445758	E2F5	E2F transcription factor 5, p130-	E2F-5

			binding	
B05	Hs.76753	ENG	Endoglin	CD105/END/FLJ41744/HHT1/ORW/ORW1
B06	Hs.517517	EP300	E1A binding protein p300	KAT3B/p300
B07	Hs.264887	FGFR1	Fibroblast growth factor receptor 1	BFGFR/CD331/CEK/FGFBR/FLG/FLJ99988/FLT2/HBGFR/KAL2/N-SAM/OGD
B08	Hs.533683	FGFR2	Fibroblast growth factor receptor 2	BEK/BFR-1/CD332/CEK3/CFD1/ECT1/FLJ98662/JWS/K-SAM/KGFR/TK14/TK25
B09	Hs.1420	FGFR3	Fibroblast growth factor receptor 3	ACH/CD333/CEK2/HSFGFR3EX/JTK4
B10	Hs.165950	FGFR4	Fibroblast growth factor receptor 4	CD334/JTK2/MGC20292/TKF
B11	Hs.94234	FZD1	Frizzled homolog 1 (Drosophila)	DKFZp564G072/FLJ95923
B12	Hs.142912	FZD2	Frizzled homolog 2 (Drosophila)	-
C01	Hs.40735	FZD3	Frizzled homolog 3 (Drosophila)	Fz-3/hFz3
C02	Hs.19545	FZD4	Frizzled homolog 4 (Drosophila)	CD344/EVR1/FEVR/FZD4S/Fz-4/FzE4/GPCR/MGC34390
C03	Hs.17631	FZD5	Frizzled homolog 5 (Drosophila)	C2orf31/DKFZp434E2135/HFZ5/MGC129692
C04	Hs.591863	FZD6	Frizzled homolog 6 (Drosophila)	Hfz6
C05	Hs.173859	FZD7	Frizzled homolog 7 (Drosophila)	FzE3
C06	Hs.302634	FZD8	Frizzled homolog 8 (Drosophila)	FZ-8/hFZ8
C07	Hs.647029	FZD9	Frizzled homolog 9 (Drosophila)	CD349/FZD3
C08	Hs.632702	GLI1	GLI family zinc finger 1	GLI
C09	Hs.111867	GLI2	GLI family zinc finger 2	HPE9/THP1/THP2
C10	Hs.21509	GLI3	GLI family zinc finger 3	ACLS/GCPS/PAP-A/PAPA/PAPA1/PAPB/PHS/PPDIV
C11	Hs.532082	IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	CD130/CDw130/GP130/GP130-RAPS/IL6R-beta
C12	Hs.555947	LEF1	Lymphoid enhancer-binding factor 1	DKFZp586H0919/TCF1ALPHA
D01	Hs.133421	LIFR	Leukemia inhibitory factor receptor alpha	CD118/FLJ98106/FLJ99923/LIF-R/SJS2/STWS/SWS
D02	Hs.6347	LRP5	Low density lipoprotein receptor-related protein 5	BMND1/EVR1/EVR4/HBM/LR3/LRP7/OPPG/OPS/OPTA1/VBCH2
D03	Hs.584775	LRP6	Low density lipoprotein receptor-related protein 6	ADCAD2/FLJ90062/FLJ90421
D04	Hs.713533	LTBP1	Latent transforming growth factor beta binding protein 1	MGC163161
D05	Hs.512776	LTBP2	Latent transforming growth factor beta binding protein 2	C14orf141/LTBP3/MSTP031
D06	Hs.289019	LTBP3	Latent transforming growth factor beta binding protein 3	DKFZp586M2123/FLJ33431/FLJ39893/FLJ42533/FLJ44138/FLJ45576/LTBP-3/LTBP2/pp6425
D07	Hs.466766	LTBP4	Latent transforming growth factor beta binding protein 4	FLJ46318/FLJ90018/LTBP-4/LTBP-4L
D08	Hs.517249	NCSTN	Nicastrin	APH2/KIAA0253
D09	Hs.371987	NFAT5	Nuclear factor of activated T-cells 5, tonicity-responsive	KIAA0827/NF-AT5/NFATL1/NFATZ/OREBP/T

				ONEBP
D10	Hs.534074	NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	MGC138448/NF-ATC/NFAT2/NFATc
D11	Hs.713650	NFATC2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	NFAT1/NFATP
D12	Hs.632209	NFATC3	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	NFAT4/NFATX
E01	Hs.77810	NFATC4	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	NF-ATc4/NFAT3
E02	Hs.495473	NOTCH1	Notch homolog 1, translocation-associated (Drosophila)	TAN1/hN1
E03	Hs.487360	NOTCH2	Notch homolog 2 (Drosophila)	AGS2/hN2
E04	Hs.8546	NOTCH3	Notch homolog 3 (Drosophila)	CADASIL/CASIL
E05	Hs.436100	NOTCH4	Notch homolog 4 (Drosophila)	FLJ16302/INT3/MGC74442/NOTCH3
E06	Hs.3260	PSEN1	Presenilin 1	AD3/FAD/PS1/S182
E07	Hs.25363	PSEN2	Presenilin 2 (Alzheimer disease 4)	AD3L/AD4/PS2/STM2
E08	Hs.534465	PSENEN	Presenilin enhancer 2 homolog (C. elegans)	MDS033/MSTP064/PEN-2/PEN2
E09	Hs.494538	PTCH1	Patched homolog 1 (Drosophila)	BCNS/FLJ26746/FLJ42602/HPE7/NBCCS/PTC/PTC1/PTCH/PTCH11
E10	Hs.202355	PTCHD2	Patched domain containing 2	DISP3/KIAA1337
E11	Hs.533597	PYGO2	Pygopus homolog 2 (Drosophila)	1190004M21Rik/FLJ33226
E12	Hs.207745	RBL1	Retinoblastoma-like 1 (p107)	CP107/MGC40006/PRB1/p107
F01	Hs.513609	RBL2	Retinoblastoma-like 2 (p130)	FLJ26459/P130/Rb2
F02	Hs.248217	RBPJL	Recombination signal binding protein for immunoglobulin kappa J region-like	RBP-L/RBPSUHL/SUH/SUHL
F03	Hs.271277	RGMA	RGM domain family, member A	RGM
F04	Hs.604588	SMAD1	SMAD family member 1	BSP1/JV4-1/JV41/MADH1/MADR1
F05	Hs.12253	SMAD2	SMAD family member 2	JV18/JV18-1/MADH2/MADR2/MGC22139/MGC34440/hMAD-2/hSMAD2
F06	Hs.714621	SMAD3	SMAD family member 3	DKFZp586N0721/DKFZp686J10186/HSPC193/HsT17436/JV15-2/MADH3/MGC60396
F07	Hs.75862	SMAD4	SMAD family member 4	DPC4/JIP/MADH4
F08	Hs.167700	SMAD5	SMAD family member 5	DKFZp781C1895/DKFZp781O1323/Dwfc/JV5-1/MADH5
F09	Hs.153863	SMAD6	SMAD family member 6	HsT17432/MADH6/MADH7
F10	Hs.465087	SMAD7	SMAD family member 7	CRCS3/FLJ16482/MADH7/MADH8
F11	Hs.123119	SMAD9	SMAD family member 9	MADH6/MADH9/SMAD8/SMAD8A/SMAD8B
F12	Hs.437846	SMO	Smoothened homolog	Gx/SMOH

			(Drosophila)	
G01	Hs.620754	SP1	Sp1 transcription factor	-
G02	Hs.463059	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	APRF/FLJ20882/HIES/MGC16063
G03	Hs.404089	SUFU	Suppressor of fused homolog (Drosophila)	PRO1280/SUFUH/SUFUXL
G04	Hs.573153	TCF7	Transcription factor 7 (T-cell specific, HMG-box)	FLJ36364/MGC47735/TCF-1
G05	Hs.516297	TCF7L1	Transcription factor 7-like 1 (T-cell specific, HMG-box)	TCF-3/TCF3
G06	Hs.593995	TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)	TCF-4/TCF4
G07	Hs.494622	TGFBR1	Transforming growth factor, beta receptor 1	AAT5/ACVRLK4/ALK-5/ALK5/LDS1A/LDS2A/SKR4/TGFR-1
G08	Hs.604277	TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)	AAT3/FAA3/LDS1B/LDS2B/MFS2/RIIC/TAAD2/TGFR-2/TGFbeta-RII
G09	Hs.482390	TGFBR3	Transforming growth factor, beta receptor III	BGCAN/betaglycan
G10	Hs.446350	TGFBRAP1	Transforming growth factor, beta receptor associated protein 1	TRAP-1/TRAP1
G11	Hs.99477	VANGL2	Vang-like 2 (van gogh, Drosophila)	KIAA1215/LPP1/LTAP/MGC119403/MGC119404/STB1/STBM/STBM1
G12	Hs.34871	ZEB2	Zinc finger E-box binding homeobox 2	FLJ42816/KIAA0569/SIP-1/SIP1/SMADIP1/ZFHX1B
H01	Hs.534255	B2M	Beta-2-microglobulin	-
H02	Hs.412707	HPRT1	Hypoxanthine phosphoribosyltransferase 1	HGPRT/HPRT
H03	Hs.523185	RPL13A	Ribosomal protein L13a	-
H04	Hs.592355	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD/GAPD/MGC88685
H05	Hs.520640	ACTB	Actin, beta	PS1TP5BP1
H06	N/A	HGDC	Human Genomic DNA Contamination	HIGX1A
H07	N/A	RTC	Reverse Transcription Control	RTC
H08	N/A	RTC	Reverse Transcription Control	RTC
H09	N/A	RTC	Reverse Transcription Control	RTC
H10	N/A	PPC	Positive PCR Control	PPC
H11	N/A	PPC	Positive PCR Control	PPC
H12	N/A	PPC	Positive PCR Control	PPC

DSS COLITIS

Mice weighing around 25-30 grams were chosen and placed in cages, two to a cage. They were fed with commercial mouse chow and received water *ad libitum*. Colitis was induced by mixing dextran sodium sulphate (Cat No. 160110, MP Biomedicals, Germany) in a concentration of 4% in the feed. Although DSS colitis is usually induced by adding DSS to the water given to the animal, we have noticed in earlier studies from this laboratory that adding DSS to the feed induced colitis more reproducibly, and this method is standard in the guide's laboratory (Ahmad et al 2000, Nandakumar et al 2008, Venkatraman et al 2000 & 2003). Changes in weight and fecal occult blood were monitored daily. Faecal occult blood was tested using Hemospot strips and reagents (Corel Clinical Reagents, Goa). In this set of experiments, DSS feeding for 9 days was necessary to reproducibly induce colitis in the animals. On day 10, the animals were sacrificed and the proximal and distal colon were fixed separately in 4% paraformaldehyde and kept at 4^0C overnight to do immunohistochemistry, simultaneously in separate 1ml vials the two segments were placed in Trizol reagent and stored at -80^0 C for RNA isolation.

MUCOSAL BIOPSY COLLECTION AND HANDLING

Mucosal biopsy specimens were obtained at colonoscopy from patients with colitis and from control patients (with normal mucosa) undergoing screening colonoscopy. Segmental biopsies (1-2 bits) were obtained from the whole colon anywhere between rectum till caecum from both colitic areas and from adjacent normal areas in order to compare the distribution of stem cells, and were immediately fixed in 4% paraformaldehyde, incubated overnight at 4°C, in order to process for immunohistochemistry. Two bits were collected from each participant in a separate vial and snap-frozen in liquid nitrogen and stored at -80° C until use.

RESULTS AND ANALYSIS

(Chapter 5)

ISOLATION AND CHARACTERIZATION OF PROLIFERATIVE CELLS FROM THE STEM CELL COMPARTMENT OF THE COLONIC EPITHELIUM

BACKGROUND

The colonic epithelium regenerates from cells located in the base of the crypt. Several techniques have been used to determine the nature and the location of the stem cells in the colonic epithelium and markers that identify these stem cells have been described. A number of factors are known to associate with epithelial cell proliferation. Traditionally these include epidermal growth factor and insulin-like growth factor. Uncharacterized factors present in foetal bovine serum also stimulate the growth of epithelial cells. Similarly colon cancer cells may produce extracellular factors that stimulate the growth of other epithelial cells. The colon harbours a very large number of bacteria, most of which are commensal in nature. These bacteria secrete soluble molecules and metabolites, some of which may be responsible for signalling to stem cells to proliferate or differentiate. In the studies described in this chapter, we attempted to isolate colonic epithelial cells from the colon of mice and maintain them in culture, to test them for ability to form clones of cells, to test whether products from certain commensal bacteria in the colon influence stem cell proliferation, and to determine the location of these stem cells along the crypt-surface axis by labelling signalling molecules in epithelial cells that are associated with differentiation of different epithelial cell lineages from colonic stem cells.

AIM

The aim of this section of the thesis was to isolate proliferative cells from the colon and subject them to assays for stem cell potential.

METHODS

1. Mouse colonocytes were isolated by EDTA chelation. Briefly, mice weighing around 28 grams were anaesthetized and the colon flushed with saline twice and everted over a plastic small thin tube, clamped proximally, distended with saline, and then clamped distally to form

an everted sac and placed it in a 50ml conical flask filled with PBS, EDTA and continuously oxygenated for 20 minutes and physiological manipulations made the colon to release colonocytes into the PBS solution, and centrifuged for a minute without any brakes and the cell pellet was resuspended in fresh PBS and counted for the assessment of viability and for further experimental procedures. Cell viability was assessed using trypan blue exclusion.

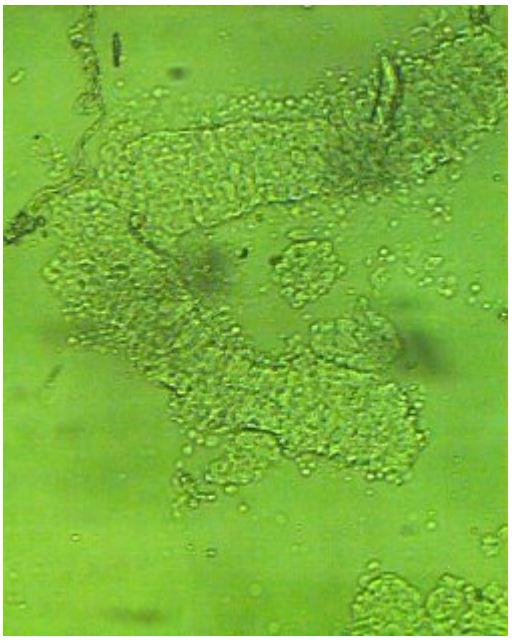
2. Clonogenic assays were done as detailed in the Methods section.
3. Signals for the isolated cells to proliferate were initially standardized by testing growth of single cell suspensions in suspension culture, and the effects of specific growth factors. The factors tested included epidermal growth factor (10ng/ml of the medium), insulin (used instead of Insulin-like Growth Factor, at conc of 0.25U/ml of the medium), spent medium of HT-29 cells (considered to contain growth factors), supernatant of *Lactobacillus plantarum*, and supernatant of *Vibrio cholerae*. Cells were scraped from each well after 14 days and total DNA was measured.

RESULTS

RESULTS OF VIABILITY AND IMMUNOCYTOCHEMISTRY

One million colonocytes were isolated from one mouse colon and their viability was 90% in trypan blue exclusion test. Colonocytes could be isolated in fractions at different time intervals of 15 minutes, 20 minutes, 30 minutes and 45 minutes fraction. We were able to get whole isolated crypts in the 45 minutes fraction. The viability of the cells, immunostaining for stem cell molecules, and PCR for mRNA expression of Musashi-1 and Hes-1 are shown in Figures in this Chapter.

Figure 5.1. Whole crypts isolated from mouse colon.



.Figure 5.2. Suspension of single cells obtained after cell isolation from mouse colon.

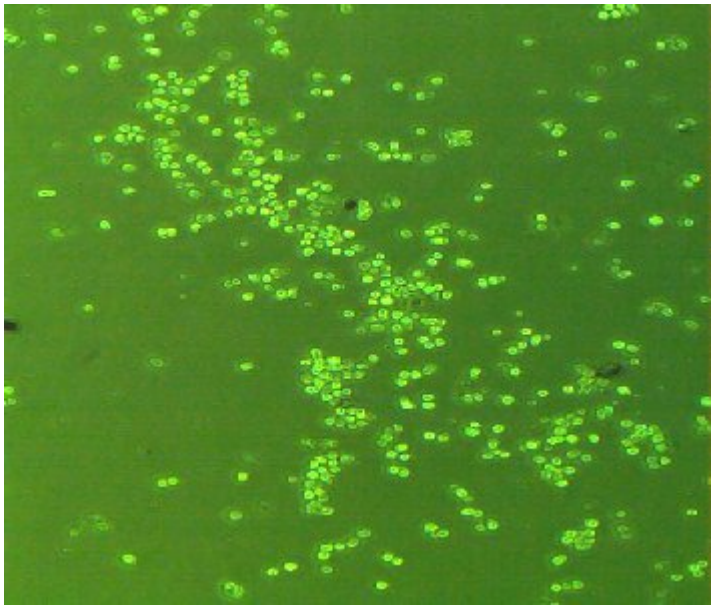


Figure 5.3. Isolated colonic epithelial cells showing immunofluorescence for beta-integrin (upper panel) and Musashi-1 (lower panel). Beta-integrin preferentially labels cells in the proliferative compartment of the colonic epithelium, while Musashi-1 preferentially labels stem cells. Nuclei are counter-stained with DAPI.

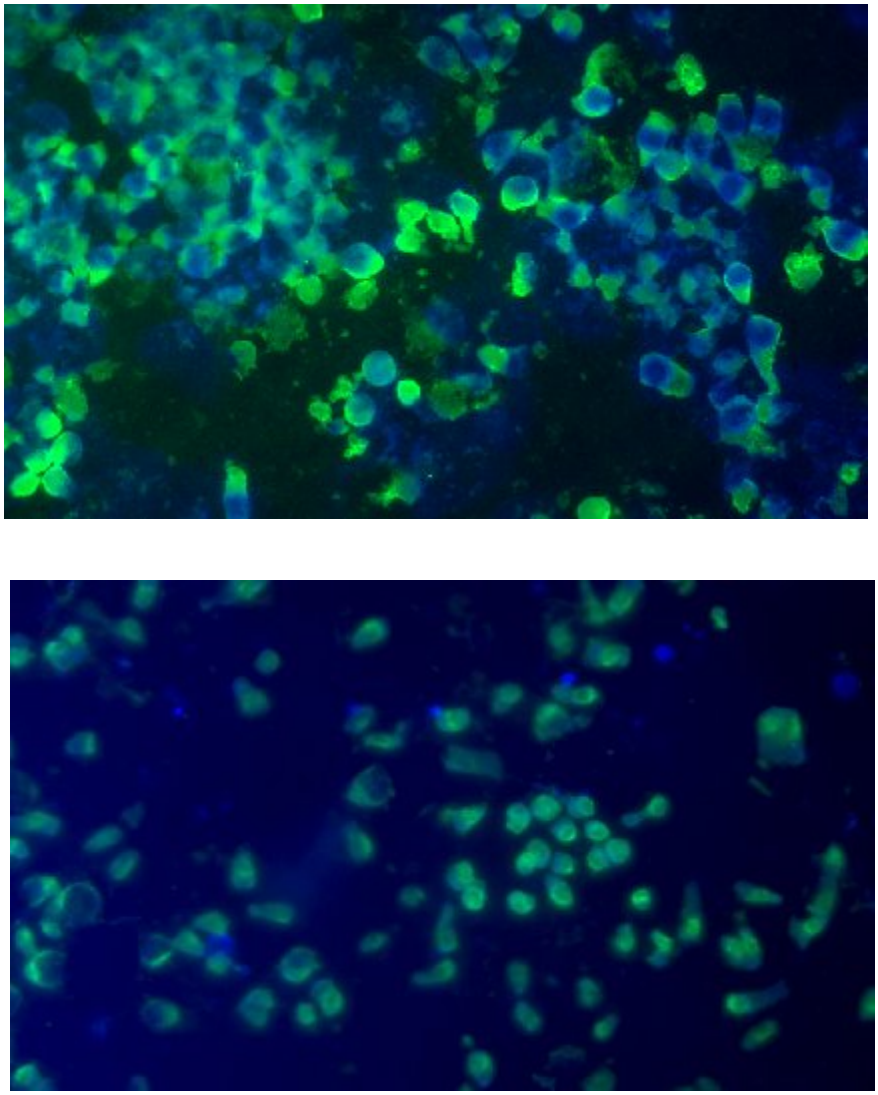
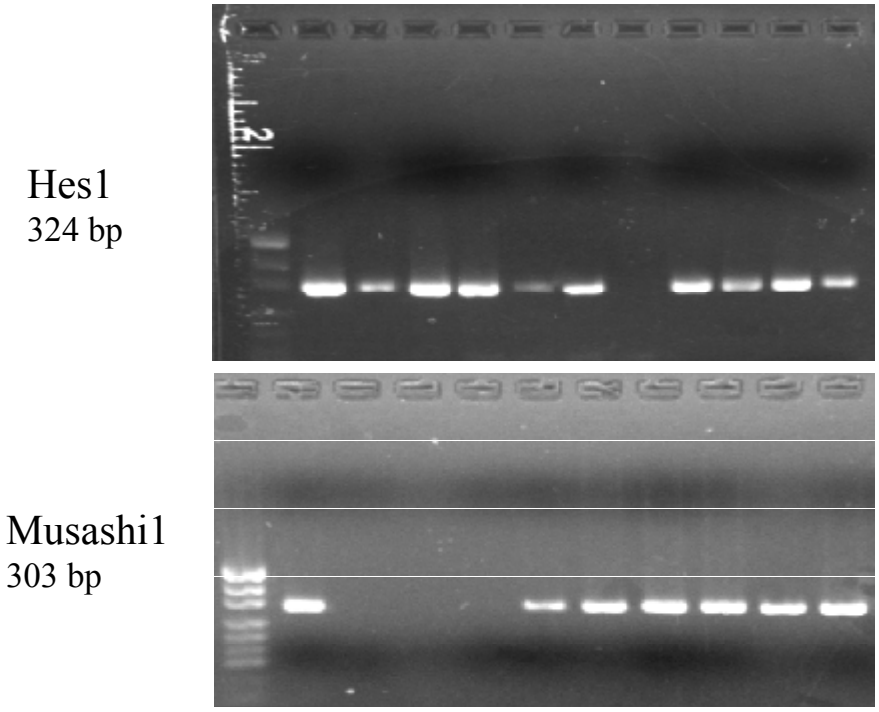


Figure 5.4. Reverse transcriptase PCR of isolated cell pellets from different extractions for Musashi 1 which is considered as a proliferative marker and Hes 1 as differentiation marker for intestinal stem cells.



RESULTS OF CLONOGENIC ASSAYS:

Clones were observed from day 5 (Figure 5.5), and kept growing in the agar in a 3 dimensional way. We could only monitor the clones which were maintained up to 70 days and could not remove them or separate them from the agar-containing 6 well plate for further analysis.

SUSPENSION CULTURE:

In suspension culture, cell aggregates formed at day 3 and were maintained till 14 days (Figure 5.6), then removed on day 14 and PCR was positive for the proliferative marker Musashi-1 and negative for the differentiation marker HES-1.

EFFECTS OF VARIOUS FACTORS ON CELL GROWTH:

The different growth factors used in this study was to hypothesize that signals derived from bacteria, or signals derived from proliferating epithelial cells could influence the proliferation of colonocytes *in vitro*. So growth factors such as EGF+Insulin, conditioned medium of *Lactobacillus* GG, HT-29, *Vibrio cholerae*, *Escherichia coli* and basal medium DMEM were used to identify which factor enhances the rate of proliferation of mouse colonic epithelial cells (Figures 5.7 and 5.8 and Tables 5.1 and 5.2). At the end of 14 days cells were removed by using cell scraper and PCR was done for the Musashi and Hes 1 (Figure 5.8). Only Musashi 1 was found positive in 14 days primary culture in all the growth factors showing that stem and progenitor cells can be kept viable in vitro in these culture conditions. As seen from the Table 5.1, cell viability was not generally affected by addition of these factors, except for Lactobacillus GG supernatant that was associated with lower epithelial cell viability. DNA content of the wells (i.e. proliferation of cells) was significantly increased only by the combination of EGF and insulin, while the other putative growth factors did not show any effect on cell proliferation of these colonic cells in this assay.

Figure 5.5. Clone seen after 28 days. 1.0% agar, double strength RPMI 1640 and 10% FBS, cells, 0.8% agarose and 10% FBS, incubated for 28 days. Cluster=15-29 cells. Clone>30 cells.

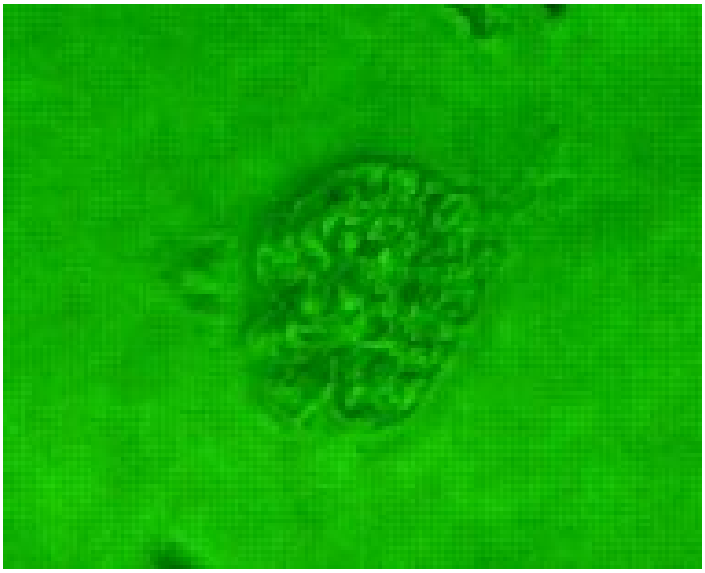
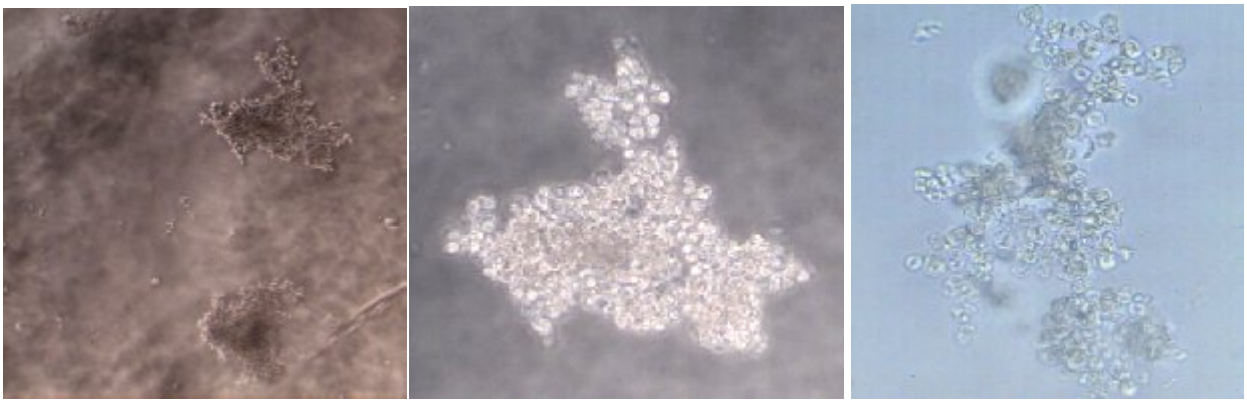


Figure 5.6. (A) Clones in Poly HEMA factor in 14 days of culture. (B) PCR for Musashi and Hes-1 on Day 14 clones. S=Sample, M=Marker (DNA ladder).



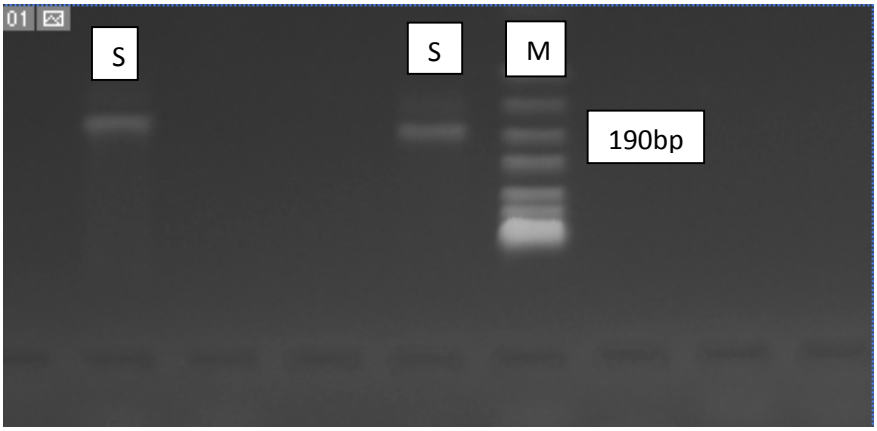


Table 5.1(a). Cell viability of isolated colonic epithelial cells seeded for suspension culture – the effect of adding putative growth factors**.**

Culture conditions	**Viability (%)**
DMEM	33
EGF + Insulin	79
HT29 supernatant	64
E. coli supernatant	68
L. rhamnosus supernatant	47
Vibrio cholerae supernatant	62

Table 5.2(a). DNA content of wells after 14 day incubation of colonocytes in culture to test effect of putative growth factors.

	DMEM	EGF + INSULIN	HT-29 conditioned	SUPERNATANT OF			
				E. coli	*L. rhamnosus*	*V. cholerae*	Initial seeding
DNA µg/well	0.38	2.12	0.21	0.45	0.25	0.21	0.13

Figure 5.7. Typical growth seen in culture after two weeks. Different conditions were studied.

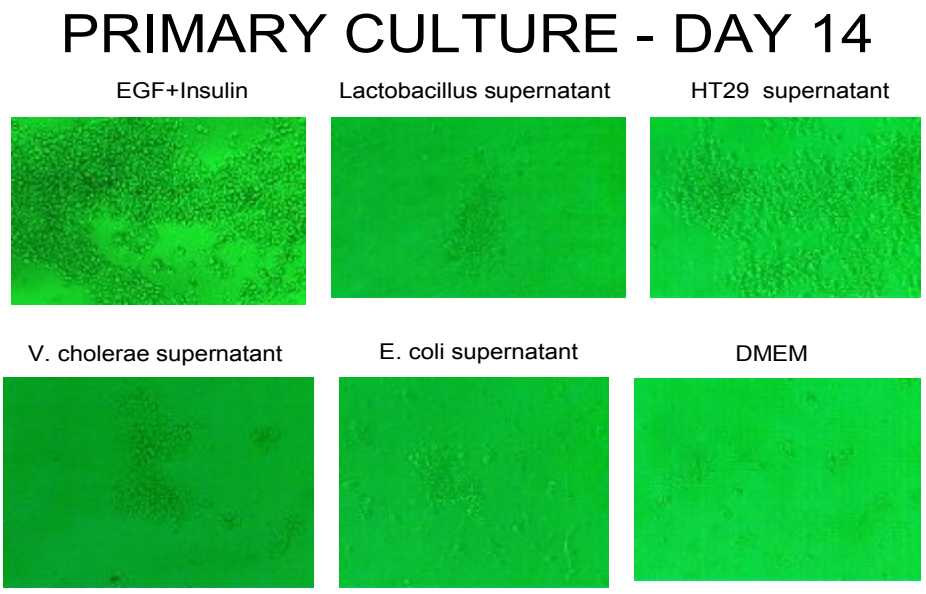
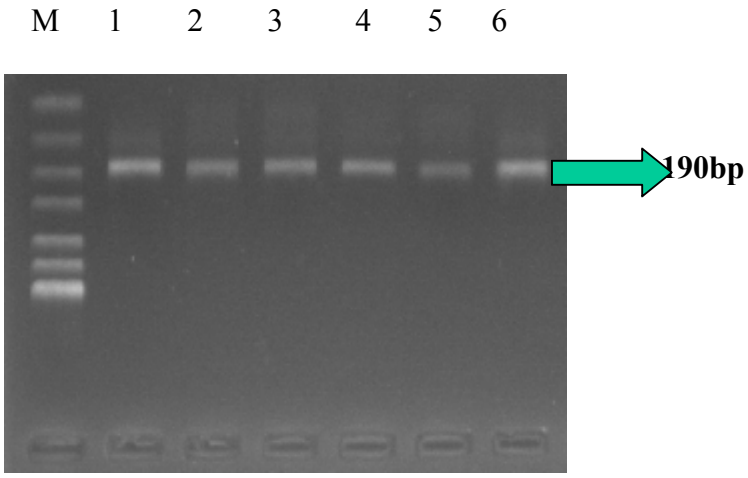


Figure 5.8. Musashi -1 mRNA expression. Lane 1-6 - Musashi -1 positive cells in14 days culture under 6 different factors.



DISCUSSION

Mouse colonocytes were isolated by EDTA chelation and fractions from this were tested for ability to proliferate and for characteristics of colonic epithelial stem cells. Stem cells characteristically have the ability to replicate in asymmetric fashion, leading to one progenitor cell and to one daughter cell that retains its stemness. One property of stem cells is their ability to remain quiescent for long periods of time. In non-stem cells, prolonged quiescence or cell cycle arrest leads to senescence and apoptosis. Stem cells possess the ability to proliferate even after long periods of quiescence. In these studies using mouse colonocytes, our primary aim was to isolate a population of colonocytes that would proliferate well *in vitro* and that would exhibit phenotypic characteristics of intestinal stem cells including presence of appropriate proteins on the cell surface.

We were able to maintain primary cultures of the isolated colonocytes for up to two weeks. The best stimulus for colonocyte proliferation appeared to be the combination of epidermal growth factor and insulin. Growth factors may potentially be released by cancer cell lines; medium conditioned by the LIM 1863 colon cancer cell line significantly increased the ability of normal proliferative colon epithelial cells to form clones (Whitehead 1999). In our experiments, however the addition of HT-29 conditioned medium did not affect proliferation of the isolated colonocytes. The colonic epithelium is in contact with numerous commensal bacteria. In the germ-free intestine, the colonic mucosal mass is small and becomes much increased only after the animal develops a bacterial flora in the intestine and colon. This suggests that factors from commensal bacteria may influence proliferation of colonic epithelial cells. However, incubation of the colonocyte cultures with supernatants of several bacteria in early log

phase did not show any increase in proliferation. If anything, it appeared that the bacterial supernatants impaired colonocyte growth.

The isolated colonic epithelial cells showed phenotypic characteristics consistent with a stem cell enriched population. Beta-integrin is a surface protein that characterizes certain stem cells including colonic epithelial stem cells. Colonic epithelial cell populations that are enriched in beta-1-integrin exhibit the ability to form clones, a characteristic of stem cells (Fujimoto 2002). The isolated colonocyte population showed a number of cells that stained for beta-integrin by immunofluorescence. Similarly, the isolated cell population also showed cells that stained for Musashi-1 protein, an RNA-binding protein that is linked to signaling and transformation of stem cells in the intestine. characterizes stem cells in several tissues including intestine (Kayahara 2003, Nishimura 2003).

The isolated cells also showed the ability to form clones when single cell suspensions were seeded in poly-Hema coated plates. This again confirms the presence of stem cells within the population of isolated colonocytes. Single cell suspensions isolated from dissociated colonic crypts were first used successfully to produce clones of epithelial cells by Whitehead and his group (Whitehead 1999). This therefore provides a mechanism to isolate proliferative epithelial cells which can be considered for use in transplantation in conditions where replacement of the colonic epithelium may be considered desirable such as extensive ulceration in the colon with absorption of bacteria and noxious products into the bloodstream as a result of loss of the colonic mucosal barrier.

CHAPTER 6

EPITHELIAL STEM CELLS IN EXPERIMENTAL COLITIS IN MICE & EFFECTS OF ALLOGENEIC EPITHELIAL CELL TRANSPLANTATION

BACKGROUND

Inflammatory bowel disease is characterized by ulceration of the surface epithelium and chronic and acute inflammation in the lamina propria of the intestine and colon. The role of the crypt cells in colitis is a little unclear and it is likely that the stem cell regeneration is increased and that proliferating cells downstream of stem cells are increased in number in order to compensate for the surface epithelial cell loss. The nature of the stem cell signaling events that occur during this change is not completely understood. Since epithelial cells are lost, it is possible that there is an exposure of adhesion molecules in the underlying connective tissue that will allow exogenously transplanted epithelial cells to adhere and grow rather in the nature of a skin transplant covering burns injuries. The studies described in this Chapter were designed to determine whether such exogenously transplanted epithelial cells would adhere to and proliferate on top of an ulcerated mucosa in experimental colitis in mice.

AIMS

1. To determine the number and distribution of epithelial stem cells expressing Musashi-1 and Hes-1 in the colon of normal mice and mice with dextran sodium sulfate-induced colitis.
2. To determine whether infusion of exogenous (allogeneic) colonic epithelial cell suspensions into the colon of mice with dextran sodium sulfate colitis would allow these epithelial cells to seed and grow in the recipient colon and alter mucosal repair.

METHODS

Colitis was induced in female Swiss albino mice by the administration of 4% dextran sodium sulfate (DSS) (Cat.No. 160110, MW36000–50000, MP Biomedicals, Illkirch, France) in the feed daily for 9 days. Preliminary experiments showed that this was the duration of acute DSS feeding that was necessary to reproducibly produce colitis in the current colony of animals. Water was

provided *ad libitum* along with the commercial mice chow. Mice were weighed every day and rectal blood loss was detected by testing the stool using Hemospot kits (Corel Clinical Systems, Goa, India) daily. Colonocytes were isolated from male Swiss albino mice as described in an earlier Chapter, and 1 ml of single cell suspension containing approximately 50,000 proliferative colonic epithelial cells were introduced by enema into the rectum of the animals with colitis. The animals were sacrificed, the colon removed, and fixed in buffered formalin and processed for light microscopy. Bits of proximal and distal colon from animals with colitis as also from control animals were also fixed in paraformaldehyde for immunohistochemistry, which was done using Musashi-1 and Hes-1 as described in the Methods Chapter.

RESULTS

Time course of development of colitis

Feeding of DSS induced colitis reproducibly, as marked by loss of weight and detection of occult blood in the stool of the DSS-treated animals. Table 6.1 shows the weight changes and occult blood changes in these animals.

Table 6.1. Time course of development of colitis as evidenced by weight loss and faecal occult blood.

	Day 1	2	3	4	5	6	7	8	9
Weight in gms mean (SE)	24.4 (0.4)	23.3 (0.3)	20.8 (0.3)	20.2 (0.3)	20.3 (0.3)	19.3 (0.3)	19.3 (0.3)	19.0 (0.3)	19.0 (0.3)
Fecal occult blood	0	0	0	1+	1+-2+		1+-2+	2+-4+	4+

There was a reduction and shrinkage in the length of the colon when compared to day zero. The histology confirmed the presence of active colitis and showed an infiltration of granulocytes, macrophages and neutrophils and the total crypt architecture was lost and only the basement membrane was visible in haematoxylin and eosin staining in severe cases (Figure 6.1).

Epithelial stem cell number and distribution in healthy animals and animals with colitis

Musashi-1 and Hes-1 staining were used to identify stem cells and early progenitor cells in the normal mouse colon as well as in colitis. Musashi 1 staining of mouse distal colonic mucosa showing cytoplasmic staining of cells at the base of the crypts. Occasional nuclei at different positions along the crypt showed staining. Higher power views showed predominantly cytoplasmic expression of the protein with occasional nuclei taking up the stain. Faint expression of Hes-1 was noted in many epithelial cells, while intense nuclear staining was noted in epithelial cells along the bases and walls of the crypts, particularly in goblet cell nuclei. In control mice, Hes-1 expression was noted mainly at the bottom of the crypts, while in DSS colitis, Hes-1 expression extended up to the upper half of the crypts as well. (Figures 6.3 to 6.9).

Figure 6.1. Light micrograph of mouse colon after DSS feeding showing the characteristic changes in crypts with shortening and distortion and crypt loss, and inflammatory cell infiltration of the lamina propria. The panel on the left shows a lesser degree of damage with a few remaining intact crypts, while the panel on the right shows an area of total crypt loss. The muscularis mucosa, submucosa and muscularis propria are seen at the bottom of the picture.

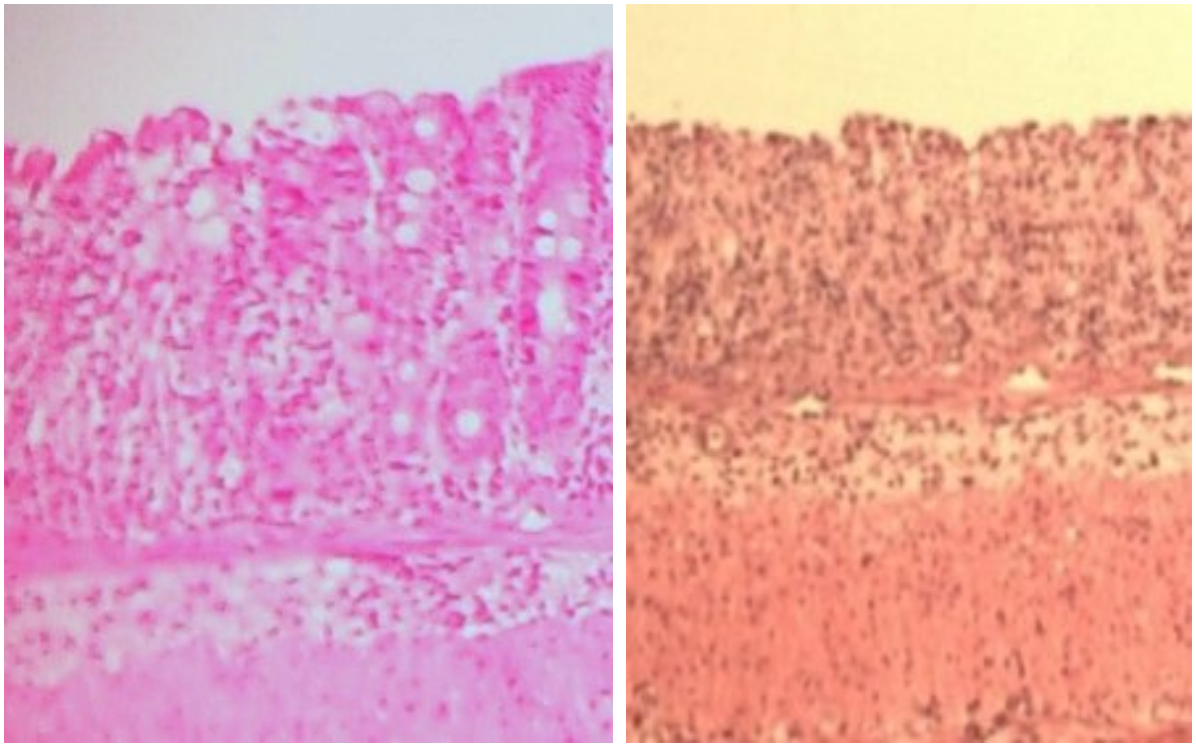


Figure 6.2. Higher power view of mouse colon after DSS showing loss of crypts and shortening of crypts with an increase in the distance between the base of the shortened crypts and the muscularis mucosa. Inflammatory cells are seen infiltrating the lamina propria beneath the base of the shortened crypts.

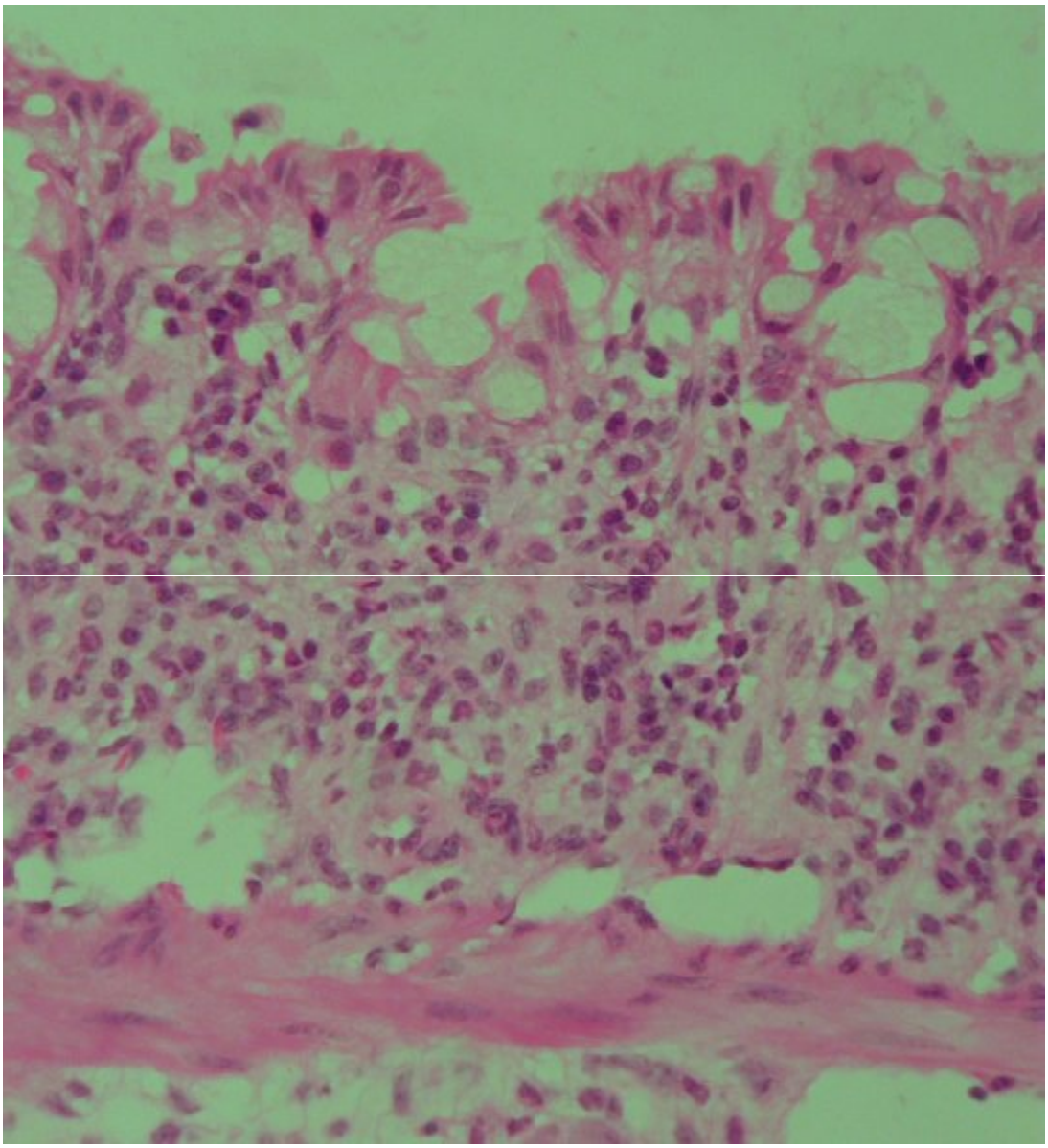


Figure 6.3. Musashi-1 staining of normal mouse distal colon showing cytoplasmic staining of cells at the base of the crypts. Occasional nuclei at different positions along the crypt show staining.

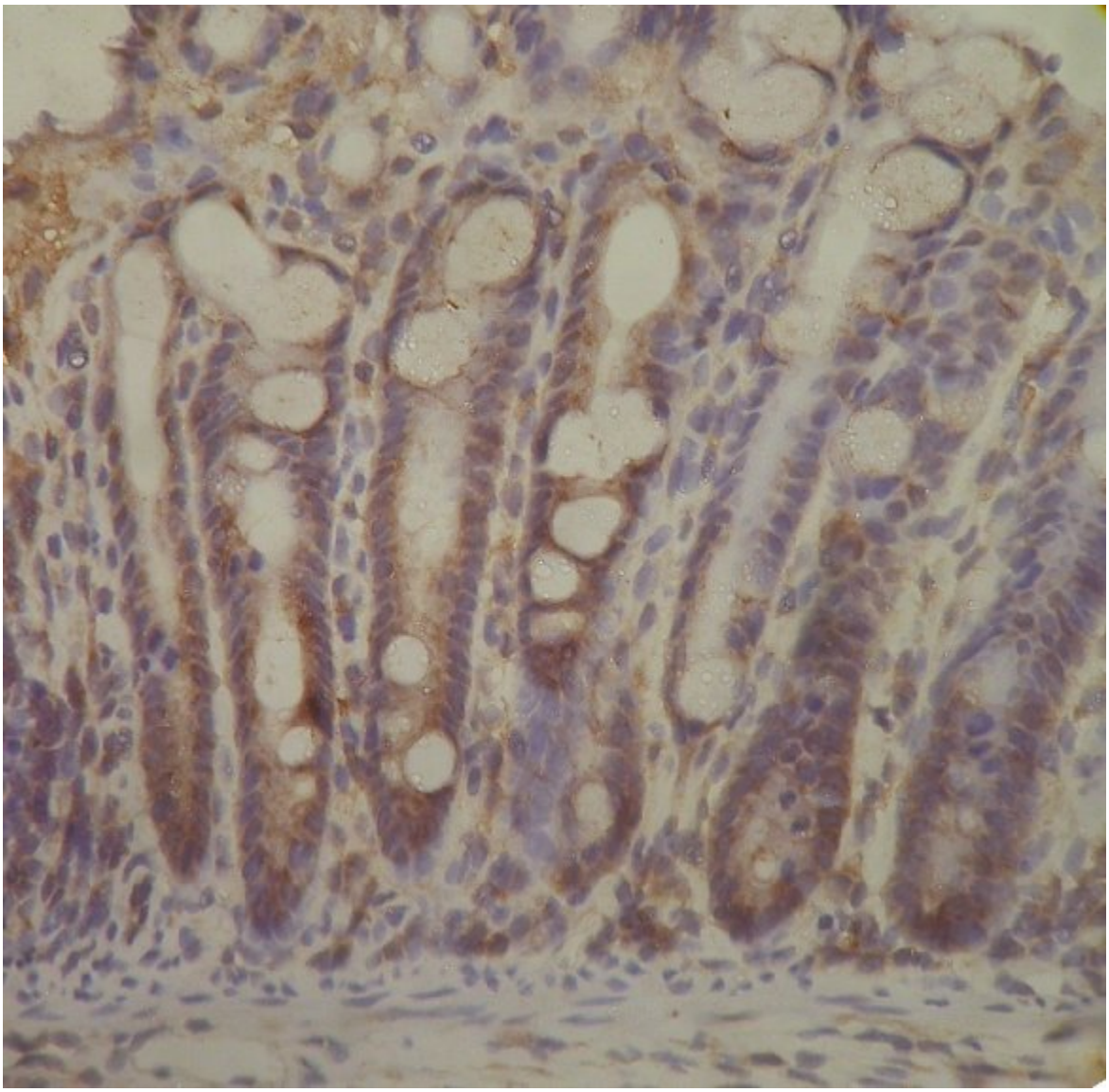


Figure 6.4. Higher power view of section of normal mouse distal colon stained for Musashi-1, showing predominantly cytoplasmic expression of the protein with occasional nuclei taking up the stain. Of note, these cells are not necessarily located at the base of the crypt.

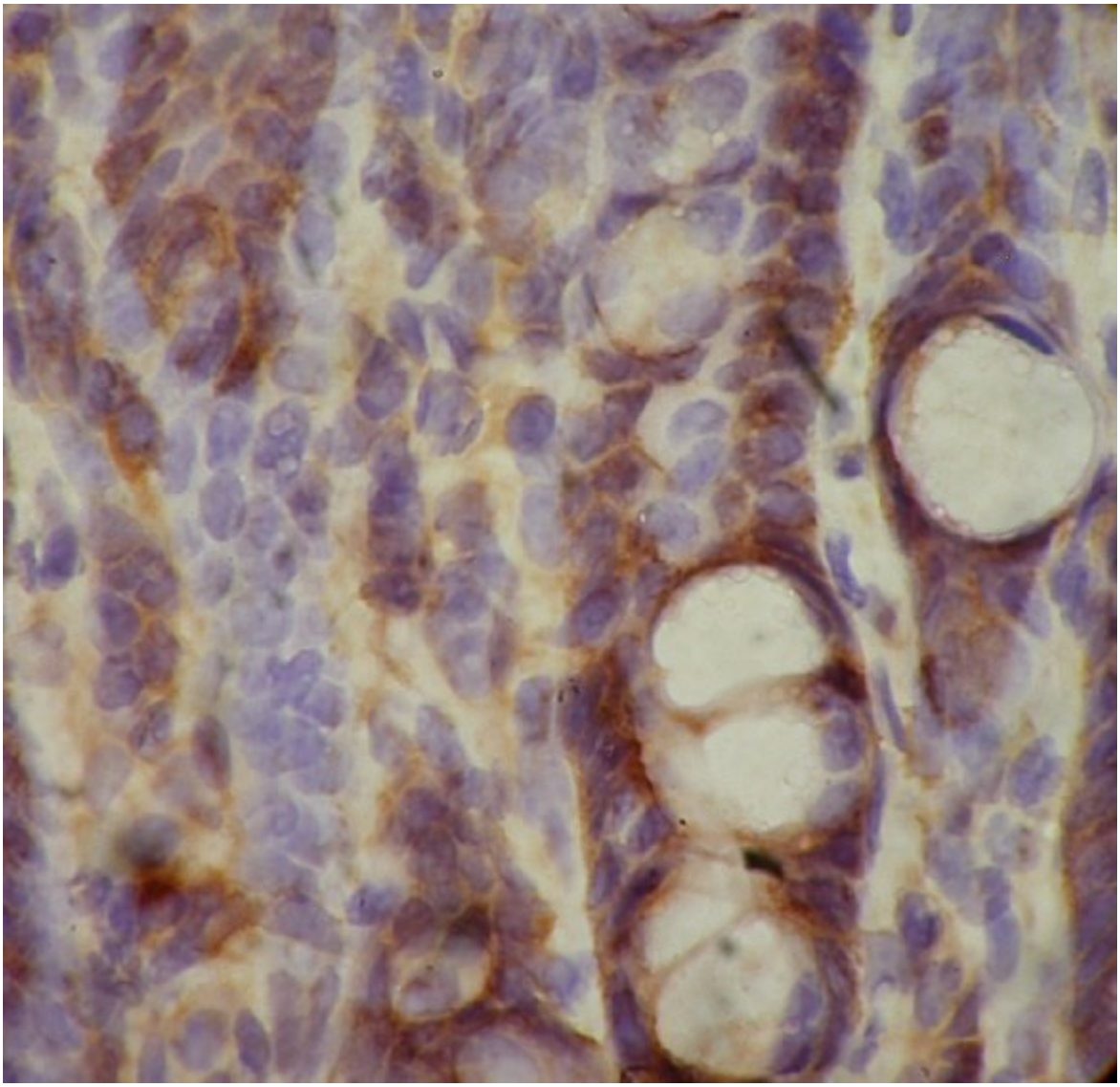


Figure 6.5. Section of mouse proximal colon immunostained for Hes-1. Faint cytoplasmic staining is noted in many epithelial cells, while intense nuclear staining is noted in epithelial cells along the bases and walls of the crypts, particularly in goblet cell nuclei.

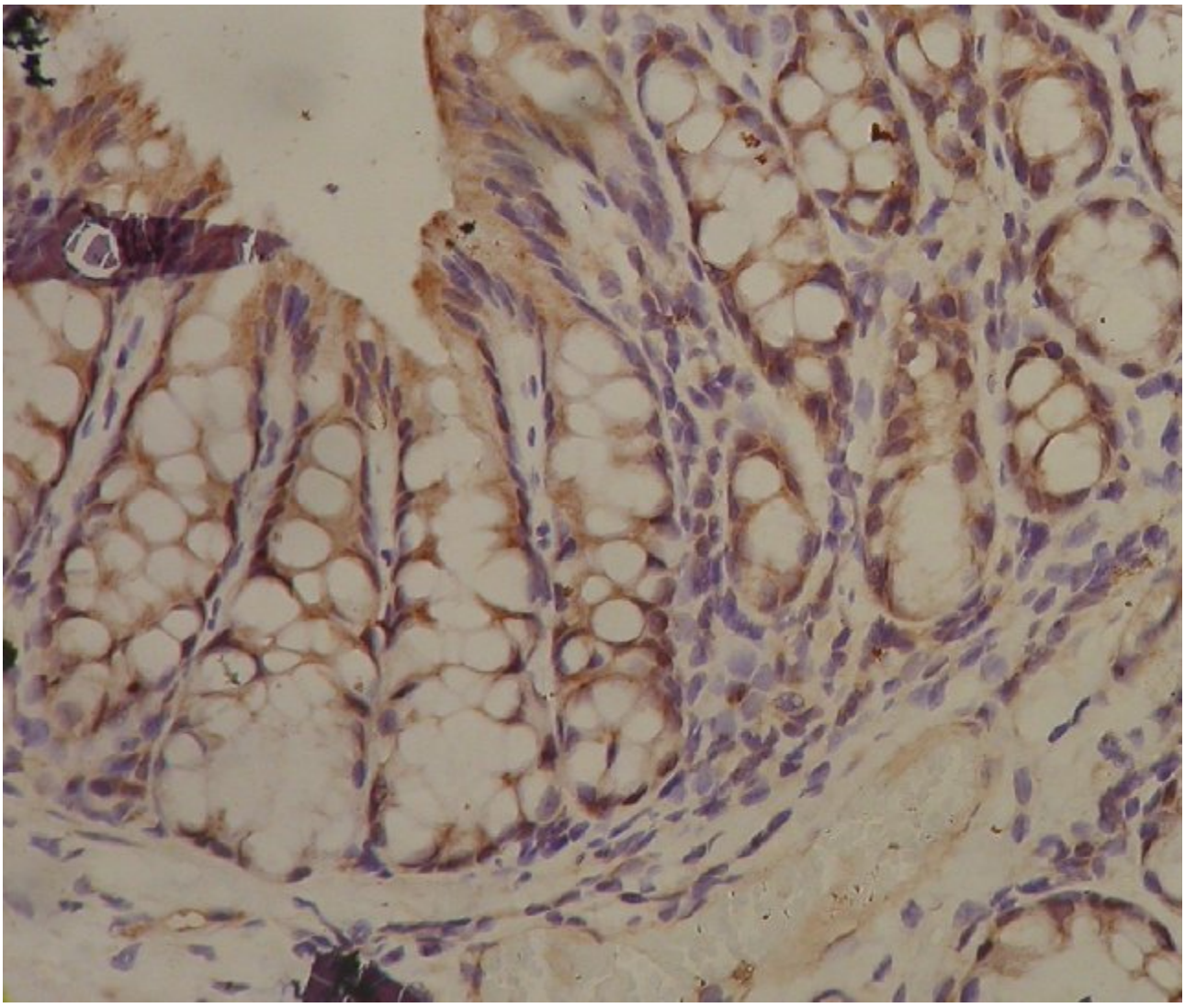


Figure 6.6. Higher power view of 6.5 showing intense staining of goblet cell nuclei along mid-crypt for Hes-1 protein.

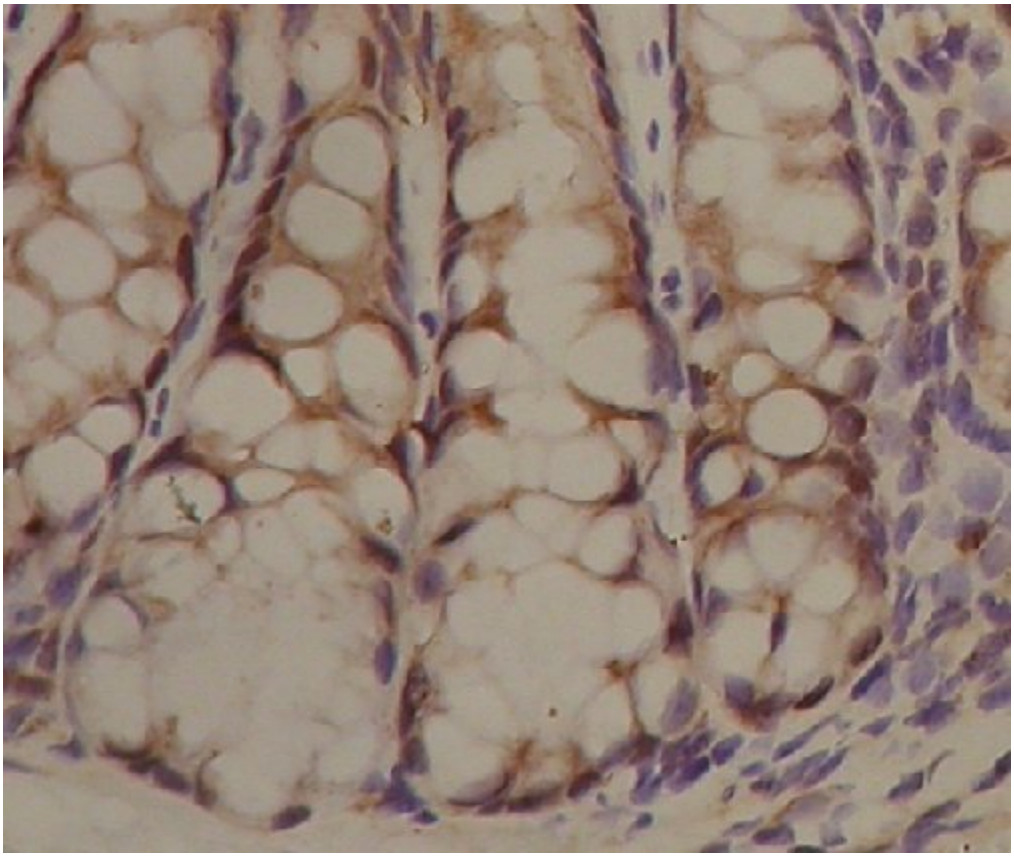


Figure 6.7. Section of normal mouse distal colon immunostained for Hes-1 showing nuclear staining of cells at the bottom of the crypts.

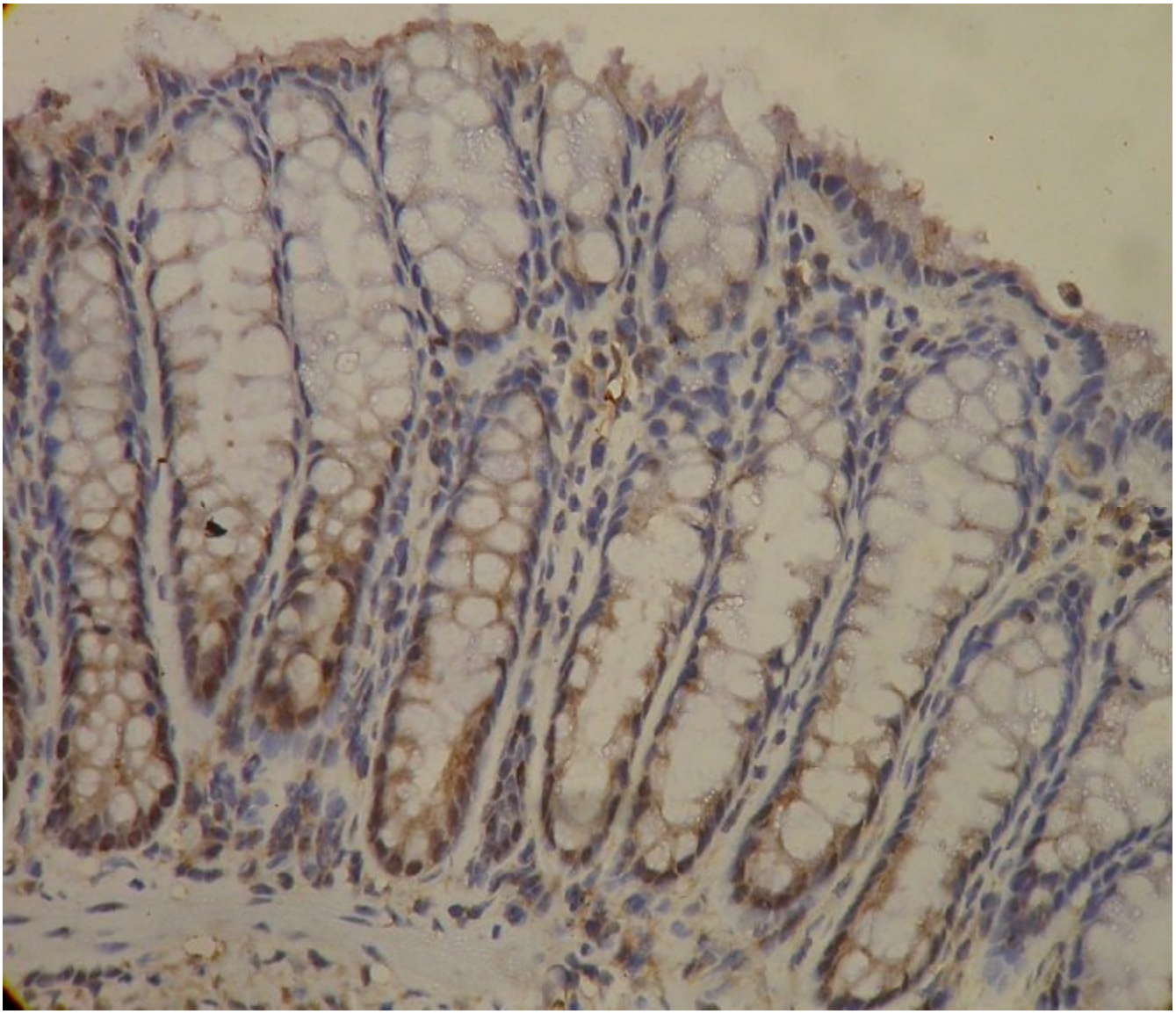


Figure 6.8. Hes-1 immunostaining of mouse proximal colon in DSS colitis. Shortening and loss of the crypts and surface ulceration is seen in this photomicrograph. Nuclear staining for Hes-1 protein is observed in the remaining crypts, especially in goblet cell nuclei.

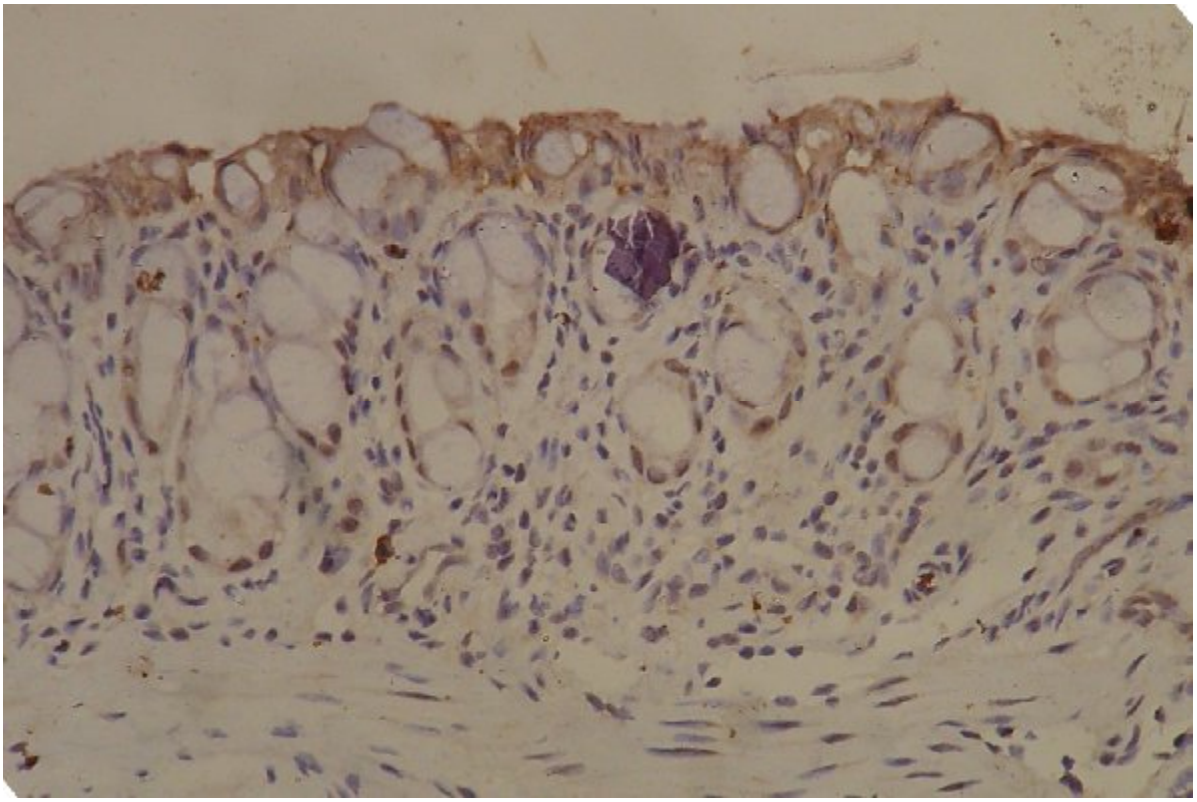
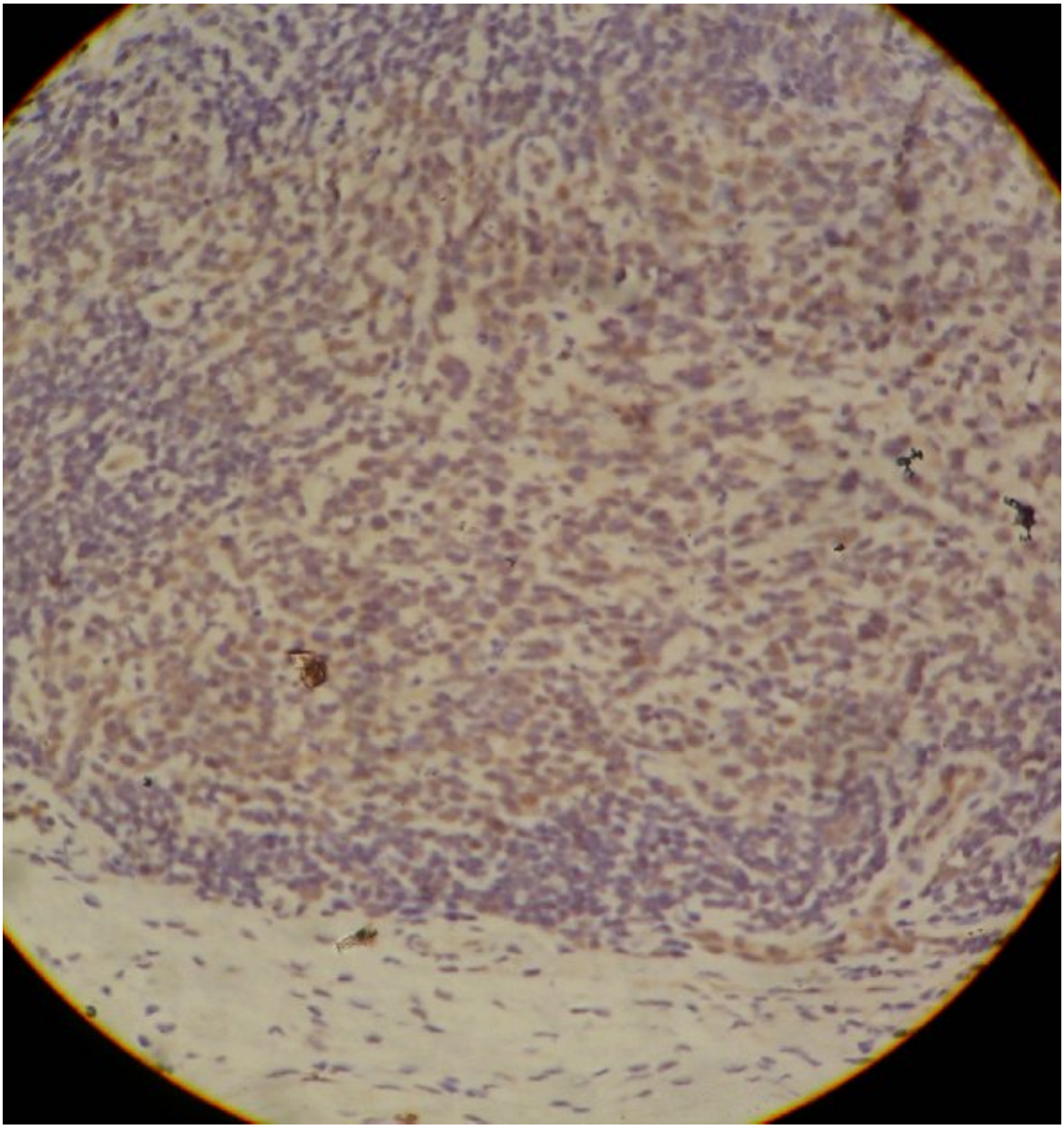


Figure 6.9. Hes-1 immunostaining of mouse distal colon in a mouse with severe DSS colitis. The upper half of the picture shows the lamina propria with total crypt loss and extensive inflammation, while the lower part represents the submucosa. Nuclear staining for Hes-1 is virtually absent in this section.



STEM CELL TRANSPLANTATION STUDIES

After the induction of colitis in female mice, colonocytes from a single male mouse were isolated and single cell suspensions containing approximately 50,000 cells of the proliferative fraction in Dulbecco's Modified Eagle's Medium were infused into the rectum of the mice with colitis on day 10. The cell infusion was performed after cleaning the colon initially with normal saline for one hour. Thereafter mice were fed normal chow for 7 days and sacrificed on the 8th day. Table 6.2 records the weight and faecal occult blood parameters in mice receiving the epithelial cell infusion into the colon and in mice receiving infusion of PBS as control into the colon.

Table 6.2. Weight change in mice with DSS colitis transplanted on Day 9 with colonocytes by luminal infusion (transplant group) or PBS (control group). Values shown are weight in grams as Mean (SE). Animals were sacrificed on Day 8 post-intervention for histological studies.

Group	Days post-infusion of colonocytes or PBS						
	1	2	3	4	5	6	8
Transplant N=6	22.2 (0.6)	22.5 (0.5)	23.5 (0.5)	23.1 (0.5)	23.5 (0.7)	23.1 (0.8)	23.4 (0.6)
Control N=5	21.3 (1.1)	20.5 (0.9)	21.7 (1.2)	21.1 (1.2)	22.6 (1.0)	22.0 (1.2)	22.5 (1.2)

Histological damage score was 23.2$\pm$10.5 in transplant group compared to 56.5$\pm$17.9 (mean$\pm$SE) in the control group (P=NS). Histological assessment of damage was done according to the method described in Nandakumar et al (2008).

In order to be able to detect donor colonocyte uptake and survival in the recipient, donor cells were tracked using *in situ* hybridization using fluorescent DNA probes aimed at the Y

chromosome (Y-FISH) (Figure 6.10). However, repeated studies showed that the infused colonocytes from male mice did not take in the ulcerated colonic mucosa (Figure 6.11) of the recipient female mice and the studies were therefore discontinued after several attempts.

DISCUSSION

The bulk of knowledge regarding intestinal epithelial epithelial stem cells is derived from studies on the small intestinal epithelium where the location, nature, and the signalling molecules that influence the fate of the stem cell progeny have been well described. In the small intestine, epithelial stem cells reside in the base of the crypt (about 6 per crypt) which are multipotent stem cells expressing leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) and olfactomedin-4 (Olfm4) (Van Es et al 2010). These stem cells give rise to an absorptive lineage of columnar epithelial cells that are found in the villi, and a secretory lineage comprising three kinds of cells – goblet cells, Paneth cells and enteroendocrine cells. It is not known whether such pathways also operate in the colonic epithelium, although by analogy they would be expected to do so. The colon lacks Paneth cells normally, and enteroendocrine cells are much less common than in the small intestine.

Musashi-1, an RNA-binding protein, is a stem cell defining protein in the colon as in certain other tissues (Kayahara et al 2003, Nishimura et al 2003). The normal mouse colon exhibited faint cytoplasmic staining for Musashi-1, but cells that showed nuclear staining for Musashi were few in number and located at different positions along the crypt. No significant alterations were noted in colitis.

Another signalling pathway that is very important in gut homeostasis is the Notch cascade. The Notch proteins comprise four single trans-membrane receptors that influence cell fate decisions and differentiation through cell-to-cell signalling. When the appropriate ligand

binds to Notch it induces proteolysis that releases the intracellular domain of Notch receptors (NICD). NICD translocates to the nucleus, where it binds to the transcription factor Rbpj or CBF-1 and activates target gene transcription. One of the best known targets of Notch is the Hes (hairy/enhancer of split) family of genes which function as transcriptional repressors. In the present studies, Hes-1 was found extensively in the nuclei of cells along the lower half of the crypts as well as in the nuclei of cells in the lamina propria, thus confirming that this protein is likely to play an important role in epithelial homeostasis in the colon. There were no significant alterations in Hes-1 positive cells in DSS colitis.

There are no previous studies of epithelial stem cell alterations in dextran sodium sulphate colitis. Thus this study provides a baseline for examining stem cell signalling and stem cell fate changes in experimental colitis.

Figure 6.10. Fluorescence *in situ* hybridization for the Y chromosome (Y-FISH). Control positive section from colon of male mouse showing many nuclei showing fluorescence at nuclear poles. Negative control from colon of normal female mouse showing absence of signal after Y-FISH.

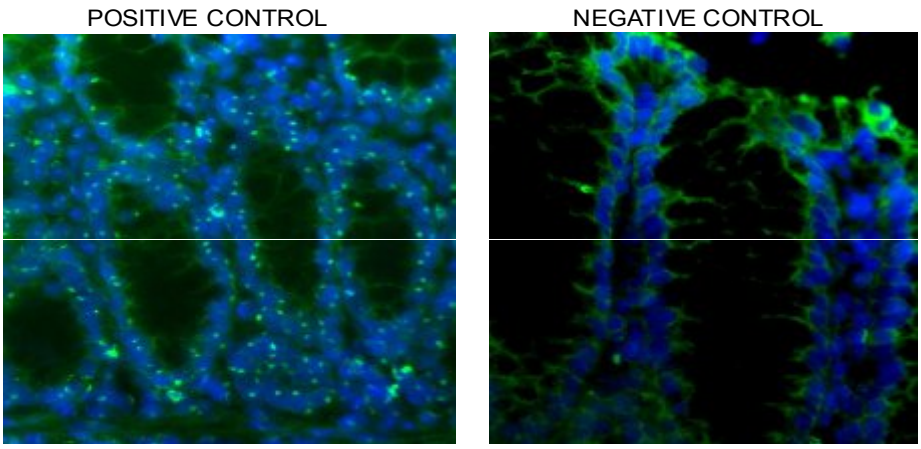
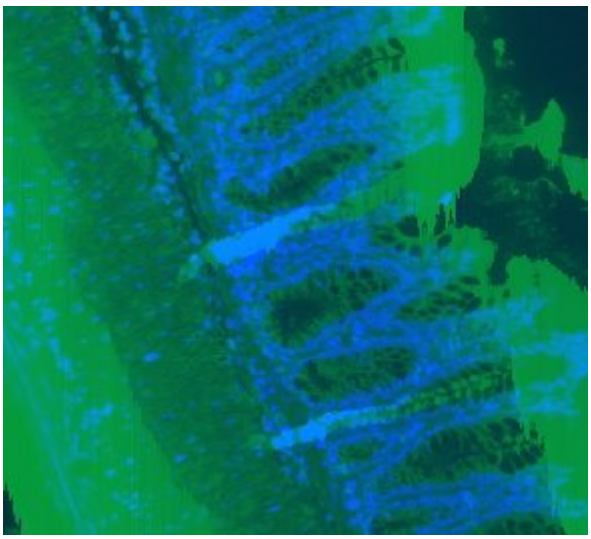


Figure 6.11. Y-FISH in a section of colon obtained from a female mouse with DSS colitis into which isolated colonic epithelial cells from a male mouse had been infused luminally via the rectum.



CHAPTER 7

EPITHELIAL STEM CELLS AND ALTERATIONS IN STEM CELL SIGNALLING IN THE COLONIC MUCOSA OF PATIENTS WITH ULCERATIVE COLITIS

BACKGROUND

Ulcerative colitis is a chronic inflammatory disorder, limited to the mucosa of the colon, that is characterized by ulceration and loss of surface epithelial cells in the colon, inflammatory changes in the crypts (cryptitis and crypt abscess) and a chronic inflammatory infiltrate in the lamina propria of the colon. In order to compensate for the loss of superficial epithelial cells, the stem cells in the crypt must regenerate more rapidly and the changes probably also involve changes in stem cell signaling. The studies in this Chapter were designed to examine this phenomenon.

AIMS

1. To determine the number and location of Musashi-1 and Hes-1 expressing epithelial proliferative cells along the crypt-surface axis of the colon in healthy individuals and patients with ulcerative colitis.
2. To determine alterations in stem cell signalling occurring in the colon of ulcerative colitis patients by comparing mRNA expression of signalling molecules in colonic biopsies from healthy individuals and patients with ulcerative colitis.

METHODS

Colonic mucosal biopsies were obtained during colonoscopy from patients with irritable bowel syndrome undergoing screening colonoscopy to exclude any colonic pathology as well as from patients with ulcerative colitis undergoing screening colonoscopy. Biopsies were fixed in paraformaldehyde, embedded in paraffin, and subjected to immunohistochemistry for Musashi-1 and Hes-1 as described in the Methods Chapter. Biopsies were taken from 16 control subjects undergoing surveillance colonoscopy and 16 patients with ulcerative colitis undergoing colonoscopy. In UC patients, colonic biopsy tissue samples were taken from

areas that were endoscopically abnormal as well as from the adjacent normal area at least 5 cm from mucosal abnormality. Biopsy bits were also snap-frozen in liquid nitrogen and stored at -80^{0}C for RNA analysis. RNA was extracted using appropriate reagents and converted to cDNA as described in the Methods Chapter. The RNA was checked and RNA from each of four healthy controls, four with absent or minimal colitis activity and four with moderate or severe colitis activity were subjected to amplification in a stem cell signalling pathway-targeted PCR array that analyzed the expression of 84 genes including stem cell-related genes and housekeeping genes. The identity and nature of the genes tested is listed in the Methods Chapter. In these biopsies it is difficult to separate the epithelial cell contribution from the contribution by inflammatory cells in the lamina propria. Hence we chose two groups of ulcerative colitis patients, those with absent or minimal inflammation who had no or few inflammatory cells in the lamina propria, and those with moderate to severe activity with large numbers of inflammatory cells in the lamina propria.

RESULTS

PATIENT CHARACTERISTICS:

16 patients (12 male) with ulcerative colitis ranged in age from 24 to 58 years (median 45). Symptoms ranged in duration from 1 month to 3 years. 7 patients had disease confined to rectum (proctitis), 2 had disease extending to sigmoid or descending colon (left-sided colitis) and the remainder had extensive disease involvement (pancolitis). Most patients received mesalazine; in addition, 3 received corticosteroids and azathioprine. Histologically, disease activity was minimal in 3, mild in 6 and moderate or severe in the remainder. The control group comprised 16 patients (10 male) with irritable bowel syndrome ranging in age from 19 to 62 years (median 38). All controls had normal colonoscopy and colonic biopsies.

MUSASHI 1 STAINING, COMPARISON BETWEEN NORMAL, MILD COLITIS, MODERATE COLITIS AND SEVERE COLITIS.

Musashi-1 protein staining was done using rabbit polyclonal anti-Musashi antibodies (AB5977, Chemicon) which react against rodent as well as human Musashi-1 protein. In colonic biopsies from normal individuals, Musashi-1 positive cells were noted mostly at the base of the crypts (Figure 7.1). In ulcerative colitis biopsies, Musashi-1-positive cells were noted in the crypt walls in the mid- and upper crypt in addition to occasional ones at the crypt base (Figure 7.2).

Quantitation of Musashi-1 cells is likely to be somewhat inaccurate since there is a shortening and deformity of crypts in the colonic mucosa in ulcerative colitis. Nevertheless, we quantitated stain-positive cells as follows. The cells were counted manually by counting the number of positive cells for Musashi per crypt in one entire tissue section and then the number of stem cells was divided by the number of total crypts in one tissue section and expressed as number of Musashi-positive cells per crypt. Figure 7.3 depicts the number of stem cells per crypt in biopsies from normal human colon, from areas of active ulceration in ulcerative colitis, and from normal-looking mucosa in ulcerative colitis. These cells were significantly fewer in number in the diseased colonic mucosa of ulcerative colitis patients ($P<0.001$, Kruskal-Wallis test with post-hoc Dunnett test) compared to normal colonic mucosa. There was no statistically significant difference in the number of cells between colonic mucosa of normal controls and normal-appearing colonic mucosa in patients with ulcerative colitis.

Figure 7.1. Sections from two normal human colon biopsies individual cells at the base of the crypts positive for Musashi-1 protein by immunohistochemistry.

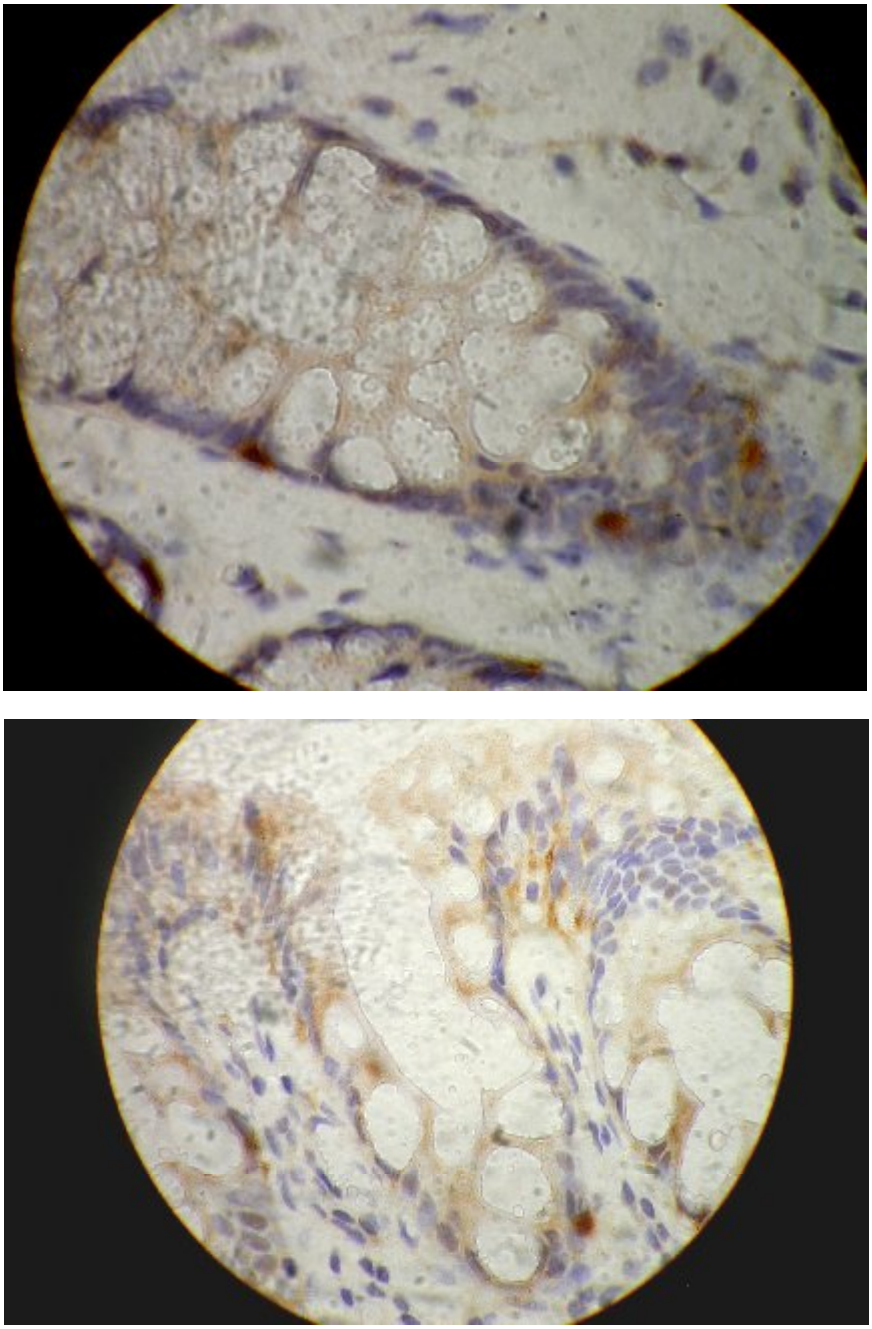


Figure 7.2. Musashi-1 staining in colonic biopsies from UC patients showing abnormal location of Musashi-1-staining stem cells away from the crypt base.

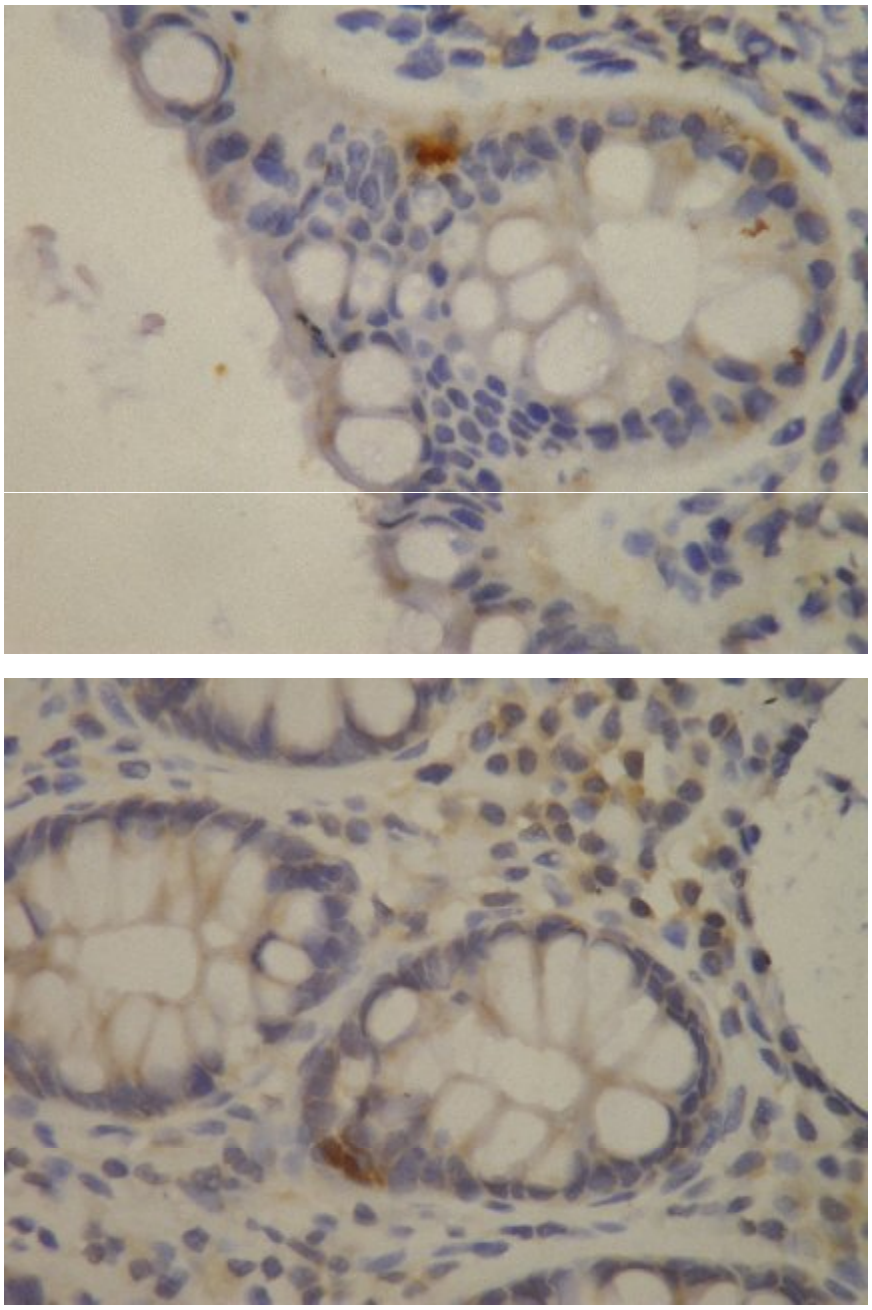
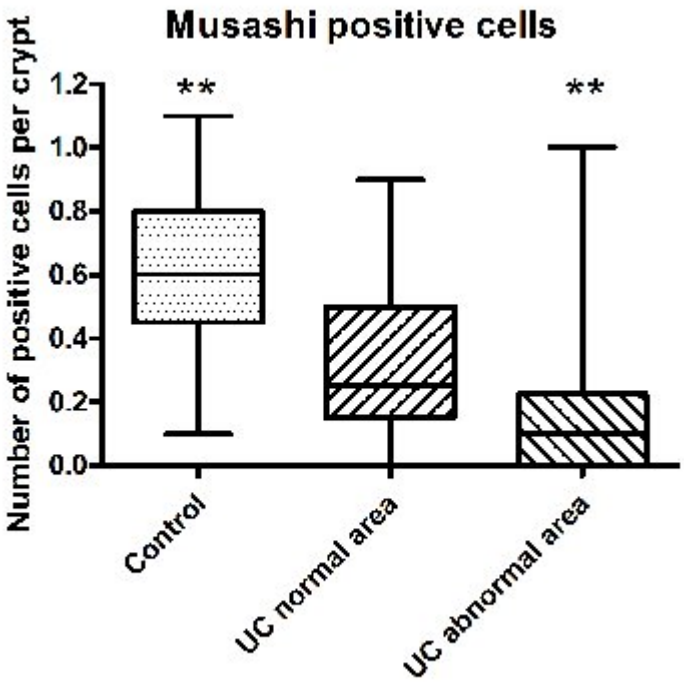


Figure 7.3. Median (with interquartile range) count of Musashi-1 positive cells in control biopsies, biopsies from normal looking mucosa in UC, and abnormal areas of mucosa in UC. P<0.001 (UC abnormal area compared to normal colon, Kruskal Wallis test with post-hoc Dunnett test).



HES-1 STAINING, COMPARISON BETWEEN NORMAL, MILD COLITIS, MODERATE COLITIS AND SEVERE COLITIS.

Hes-1 expression was similar in both control and ulcerative colitis patient samples. Epithelial cells at the base and the walls of the lower half of the crypts showed nuclear staining for Hes-1 protein (Figure 7.4). A number of cells in the lamina propria also showed nuclear staining for Hes-1 protein (Figure 7.4). In ulcerative colitis biopsies, Hes-1 positive cells were again noted in the base and side walls of the lower half of the crypts (Figures 7.5 & 7.6) and some of the epithelial cell nuclei were enlarged and distorted with a punctuate staining pattern. In some UC biopsies, Hes-1 nuclear staining was noted even in the surface epithelium. In UC biopsies, there were large numbers of lamina propria cells showing nuclear staining for Hes-1 protein (Figure 7.7).

Figure 7.4. Hes-1 protein immunostaining in colonic biopsy from normal control. Hes-1 nuclear staining is noted in many cells at the bases of the crypts as well as in some lamina propria cells.

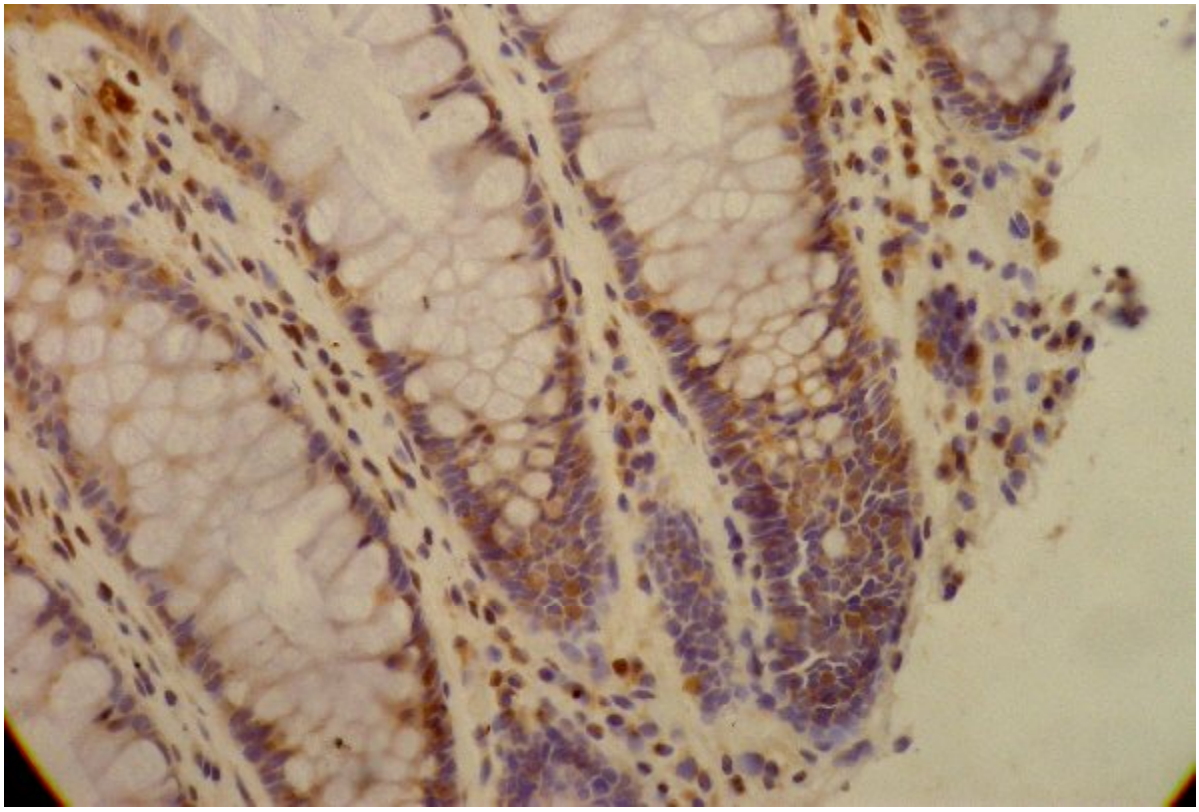


Figure 7.5. Immunohistochemistry for Hes-1 protein in colonic biopsy from patient with colitis (right panel). Note the surface ulceration and inflammatory infiltrate in the lamina propria. Nuclear staining for Hes-1 is noted in inflammatory cells in the lamina propria as well as cells all along the base and walls of the crypt all the way towards the surface in a slightly deformed crypt.

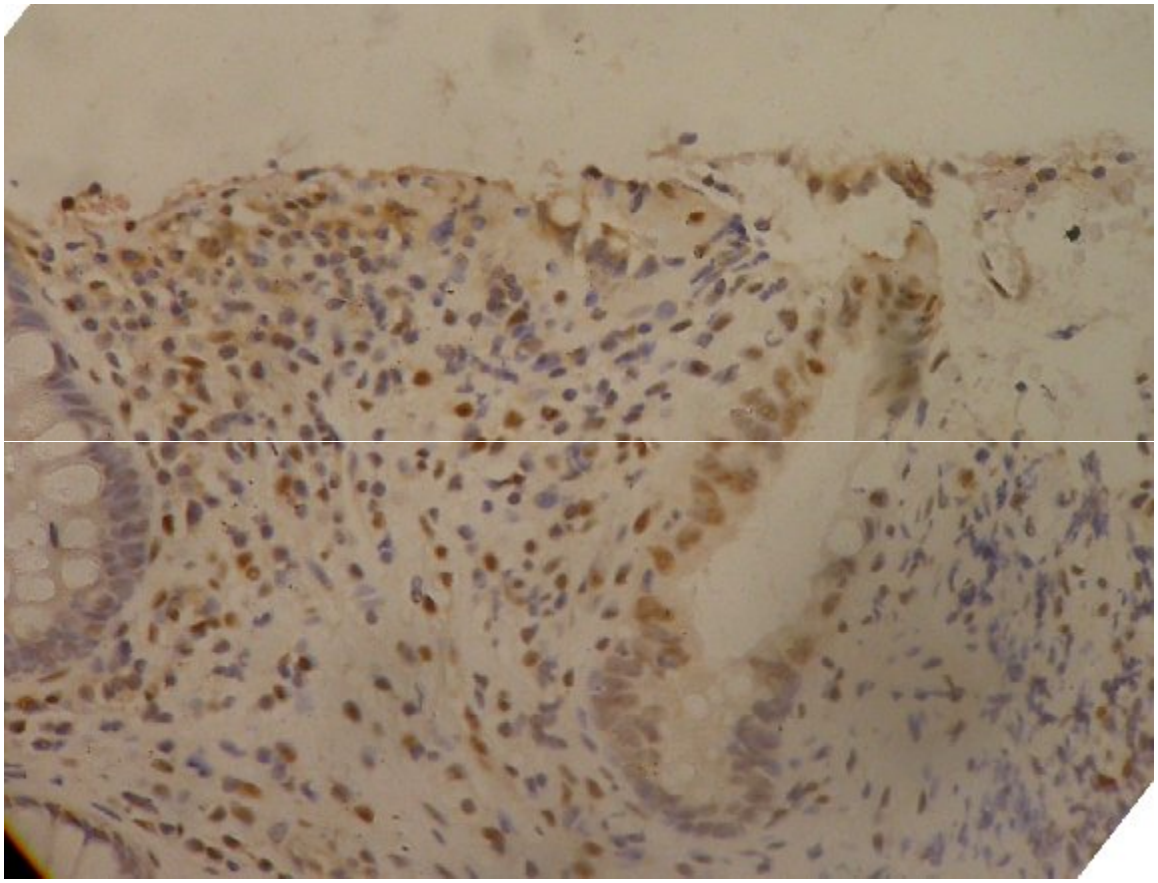


Figure 7.6. Higher power view of deformed crypt from biopsy from patient with colitis showing Hes-1 punctuate nuclear staining of epithelial cells along the base and side walls of the crypt.

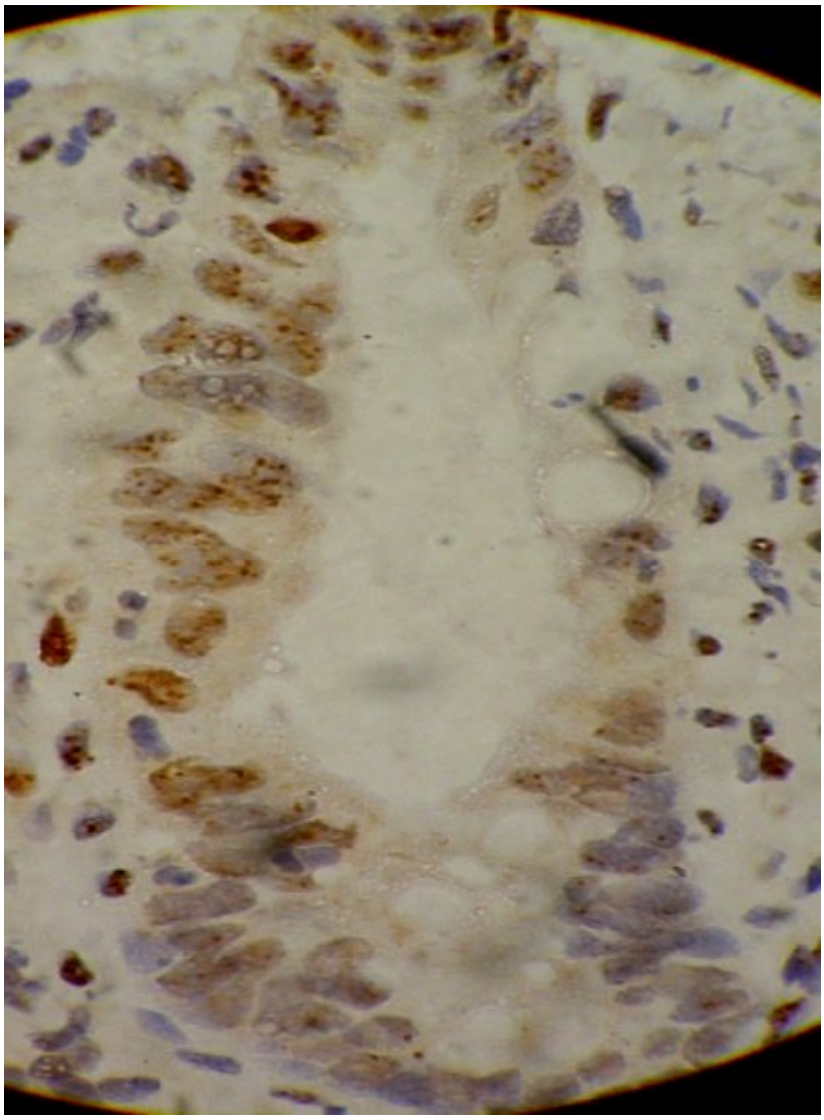
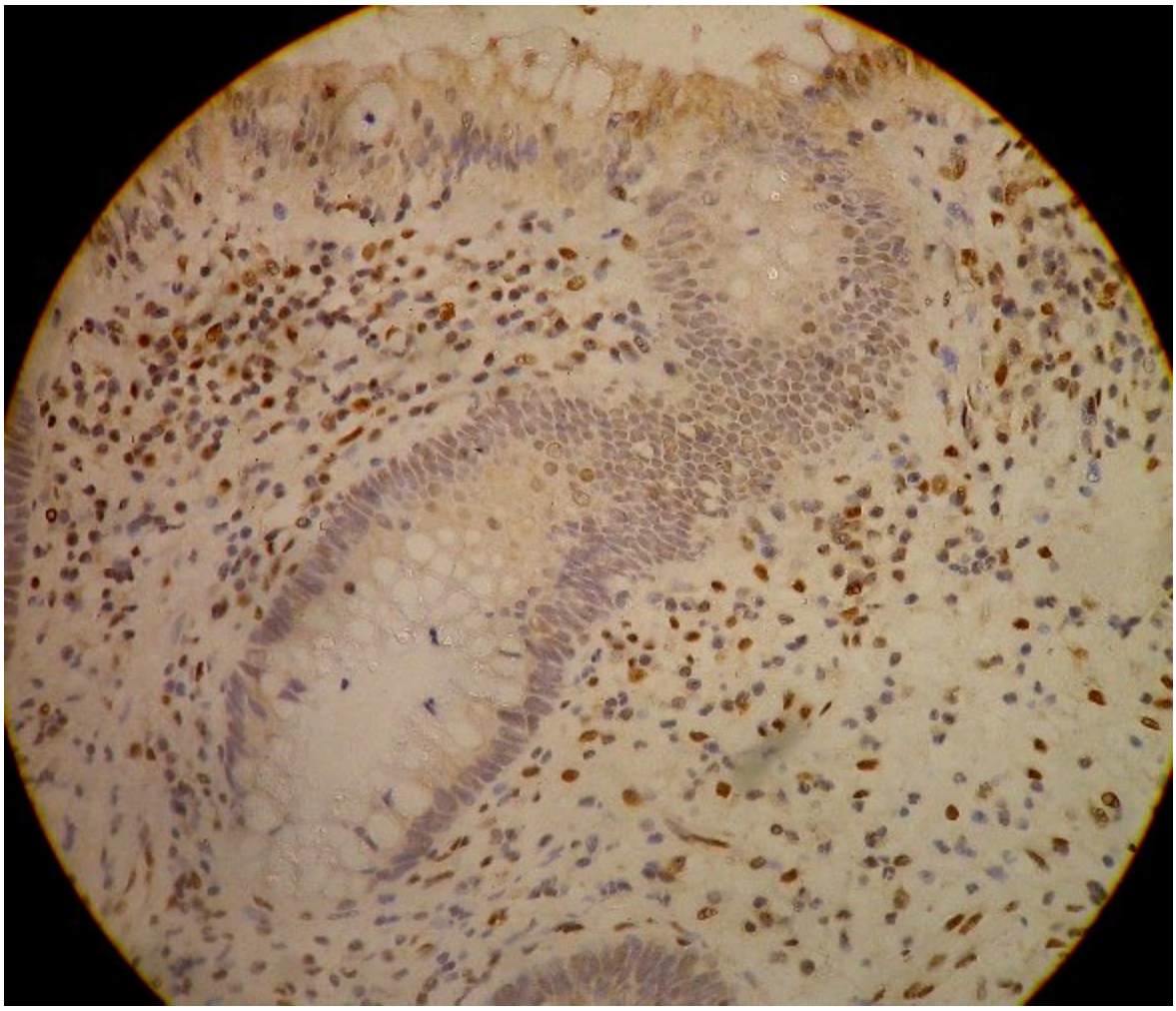


Figure 7.7. Hes-1 immunostaining of colonic biopsy from another patient with mildly active chronic ulcerative colitis showing scattered epithelial cell nuclei along the crypt walls staining for Hes-1, with numerous lamina propria cells also showing nuclear staining.



PCR ARRAY FOR ANALYSIS OF ALTERATIONS IN STEM CELL SIGNALLING

Colonic mucosal biopsies from three groups of participants were subjected to a PCR array designed to evaluate the expression of signalling genes in multipotent stem cells. The three groups included controls with a normal colonic mucosa, patients with minimal or mild ulcerative colitis with absence of significant inflammation in the lamina propria and patients with moderate to severe ulcerative colitis who had moderate to severe inflammatory infiltrate in the lamina propria. We reasoned that the contribution by inflammatory cells would be high in severe colitis, and thus chose to compare also mild colitis. Gene expression of each group was measured by real time PCR after converting the RNA to cDNA. Gene expression was expressed relative to the housekeeping genes in the array and the genes that performed best were chosen as the denominator. All gene expressions were first normalized to expression of the housekeeping genes; then gene expression in the study groups (1=mild, 2=moderate-severe) were expressed as fold change relative to the relevant gene in the control biopsies. The software automatically provides parameters of quality and variation, and identifies instances where amplification is not optimal. Fold-change (2^(- Delta Delta Ct)) is the normalized gene expression (2^(- Delta Ct)) in the Test Sample divided by the normalized gene expression (2^(- Delta Ct)) in the Control Sample. Fold-change values less than one indicate a negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change. The p values are calculated based on a Student's t-test of the replicate 2^(- Delta Ct) values for each gene in the control group and treatment groups, and p values less than 0.05 are indicated in red. The following results were noted:

Pluripotency Maintenance Signalling Genes

The expression of these genes was not altered in biopsies from patients with ulcerative colitis in either Group 1 or Group 2, indicating that these genes are not affected in the colonic mucosa in ulcerative colitis (Table 7.1).

Table 7.1. Pluripotency maintenance genes whose mRNA expression was tested. "Not altered" indicates that expression was not statistically significantly altered in relation to the control biopsies.

Gene	Description	Group 1	Group 2
IL6ST (GP130)	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	Not altered	Not altered
LIFR	Leukemia inhibitory factor receptor alpha	Not altered	Not altered
STAT3	STAT3	Not altered	Not altered

Fibroblast Growth Factor (FGF)-signalling genes:

This group of genes was differentially affected in inactive and active UC (Table 7.2). The FGFR1, which was highly down-regulated in active UC (Group 2) in this study, is found in many tissues and is involved in tissue repair, but its specific role in the intestine is uncertain. CDX2, which was highly down regulated in inactive UC (Group 1), is involved intestinal (especially colonic) differentiation. Its down-regulation in mild UC suggests that the colonic epithelial stem cells probably become less differentiated in this condition.

Table 7.2. FGF-signalling genes whose mRNA expression was tested. "Not altered" indicates that expression was not statistically significantly altered in relation to the control biopsies. The "fold-down-regulation" was calculated as described in the text. Statistically significant values ($P<0.05$) as well as values showing trend to significance ($P<0.10$ & >0.05) are shown.

Gene	Description	Group 1	Group 2
FGFR1	Fibroblast growth factor receptor 1	Not altered	10.5 fold down-regulation P=0.0427
FGFR2	Fibroblast growth factor receptor 2	Not altered	Not altered
FGFR3	Fibroblast growth factor receptor 3	Not altered	Not altered
FGFR4	Fibroblast growth factor receptor 4	Not altered	Not altered
CDX2	Caudal type homeobox 2	2.54 fold down-regulation P=0.0642	Not altered

Hedgehog signalling genes:

Sonic hedgehog and Indian hedgehog are intimately involved in the development of the normal crypt-villus axis in the small intestine (Madison et al 2005). Their role in colonic epithelial

development is not known. None of the genes concerned with Hedgehog signalling showed any alteration of expression in ulcerative colitis (Table 7.3).

Table 7.3. Sonic and Indian Hedgehog-signalling genes whose mRNA expression was tested. "Not altered" indicates that expression was not statistically significantly altered in relation to the control biopsies.

Gene	Description	Group 1	Group 2
PTCH1	Patched homolog 1 (Drosophila)	Not altered	Not altered
PTCHD2	Patched domain containing 2	Not altered	Not altered
SMO	Smoothened homolog (Drosophila)	Not altered	Not altered
GLI1	GLI family zinc finger 1	Not altered	Not altered
GLI2	GLI family zinc finger 2	Not altered	Not altered
GLI3	GLI family zinc finger 3	Not altered	Not altered
SUFU	Suppressor of fused homolog (Drosophila)	Not altered	Not altered

Notch signalling genes:

Notch signalling is an important signalling pathway in intestinal epithelial stem cell physiology. In this set of genes, only Notch3 and Notch2 were significantly down-regulated in Group 1 and were not altered in Group 2 (Table 7.4). Information regarding the expression of Notch2 in the colon is available showing that it is found in specific crypt cells in the small intestine, in lamina propria lymphocytes and in Peyer's patches in the small bowel. The role of Notch3 in the colon remains to be explored (Sander & Powell 2004, Katoh & Katoh 2007).

Table 7.4. Genes connected with Notch-signalling whose mRNA expression was tested. "Not altered" indicates that expression was not statistically significantly altered in relation to the control biopsies. The "fold-down-regulation" was calculated as described in the text. Statistically significant values ($P<0.05$) as well as values showing trend to significance ($P<0.10$ & >0.05) are shown.

Gene	Description	Group 1	Group 2
NCSTN	Nicastrin	Not altered	Not altered
NOTCH1	Notch homolog 1, translocation-associated (Drosophila)	Not altered	Not altered
NOTCH2	Notch homolog 2 (Drosophila)	2.19 fold down-regulation, P=0.0710	Not altered
NOTCH3	Notch homolog 3 (Drosophila)	4.74 fold down-regulation, P=0.0379	Not altered
NOTCH4	Notch homolog 4 (Drosophila)	Not altered	Not altered
PSENEN	Presenilin enhancer 2 homolog (C. elegans)	Not altered	Not altered
PSEN1	Presenilin 1	Not altered	Not altered
PSEN2	Presenilin 2 (Alzheimer disease 4)	Not altered	Not altered
RBPJL	Recombination signal binding protein for immunoglobulin kappa J region-like	Not altered	Not altered

TGFβ Superfamily signalling genes:

The ACVRL1 gene was significantly down-regulated in Group 1 (Table 7.5). This gene encodes a type I cell surface receptor for the TGF-β superfamily of ligands and mutations in the gene are associated with hereditary haemorrhagic telangiectasia in the colon. SMAD3 and SMAD9 were both down-regulated in both groups. SMAD3 regulates the number and position of proliferating cells in the colonic crypts and contributes to the formation of a proliferative zone at the bottom of the crypts in the normal colon through Ephrin B (Furukawa et al 2011). It is an intracellular mediator of TGF-β and its deficiency accelerates re-epithelialization of the colonic mucosa (Furukawa et al 2011). Thus, reduction of the gene product in UC probably reflects activation of re-epithelialization. SMAD3 also mediates TGF-β signalling in lymphocytes and its deficiency causes immune dysregulation and colitis in mice (Bommireddy et al 2011). SMAD7 inhibits Smad signalling by TGF-β type 1 receptor superfamily members by associating with their receptors and has been implicated in genesis of colon cancer (Halder et al 2008). None of the other genes in this group was affected in UC.

Table 7.5. Genes connected with TGFβ Superfamily-signalling whose mRNA expression was tested. "Not altered" indicates that expression was not statistically significantly altered in relation to the control biopsies. The "fold-down-regulation" was calculated as described in the text. Statistically significant values ($P<0.05$) as well as values showing trend to significance ($P<0.10$ & >0.05) are shown.

Gene	Description	Group 1	Group 2
ACVRL1	Activin A receptor type II-like 1	3.86 fold down-regulated, P=0.0449	Not altered

ACVR1	Activin A receptor, type I	Not altered	Not altered
ACVR1B	Activin A receptor, type IB	Not altered	Not altered
ACVR1C	Activin A receptor, type IC	Not altered	Not altered
ACVR2A	Activin A receptor, type IIA	Not altered	Not altered
ACVR2B	Activin A receptor, type IIB	Not altered	Not altered
AMHR2	Anti-Mullerian hormone receptor, type II	Not altered	Not altered
BMPR1A	Bone morphogenetic protein receptor, type IA	Not altered	Not altered
BMPR1B	Bone morphogenetic protein receptor, type IB	Not altered	Not altered
BMPR2	Bone morphogenetic protein receptor, type II (serine/threonine kinase)	Not altered	Not altered
ENG	Endoglin	Not altered	Not altered
LTBP1	Latent transforming growth factor beta binding protein 1	Not altered	Not altered
LTBP2	Latent transforming growth factor beta binding protein 2	Not altered	Not altered
LTBP3	Latent transforming growth factor beta binding protein 3	Not altered	Not altered
LTBP4	Latent transforming growth factor beta binding protein 4	Not altered	Not altered
RGMA	RGM domain family,	Not altered	Not altered

	member A		
TGFBR1	Transforming growth factor, beta receptor 1	Not altered	Not altered
TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)	Not altered	Not altered
TGFBR3	Transforming growth factor, beta receptor III	Not altered	Not altered
TGFBRAP1	Transforming growth factor, beta receptor associated protein 1	Not altered	Not altered
EP300	E1A binding protein p300	Not altered	Not altered
SMAD1	SMAD family member 1	Not altered	Not altered
SMAD2	SMAD family member 2	Not altered	Not altered
SMAD3	SMAD family member 3	5.58 fold down-regulated, P=0.0493	22.01 fold down-regulated, P=0.0268
SMAD4	SMAD family member 4	Not altered	Not altered
SMAD5	SMAD family member 5	Not altered	Not altered
SMAD6	SMAD family member 6	Not altered	Not altered
SMAD7	SMAD family member 7	3.45 fold down-regulated, P=0.0584	4.79 fold down-regulated, P=0.0474
SMAD9	SMAD family member 9	Not altered	Not altered
E2F5	E2F transcription factor 5, p130-binding	Not altered	Not altered
RBL1	Retinoblastoma-like 1 (p107)	Not altered	Not altered
RBL2	Retinoblastoma-like 2 (p130)	Not altered	Not altered

SP1	Sp1 transcription factor	Not altered	Not altered
ZEB2	Zinc finger E-box binding homeobox 2	Not altered	Not altered

Wnt signalling genes:

Canonical wnt signalling is important in the stem cell niche in the colon and has a very broad role in maintaining gene expression in the adult small intestine and colon (Kuhnert et al 2003). Inhibition of this pathway leads to loss of proliferative crypts with eventual colonic inflammation (Hoffmann et al 2004). FZD7 and FZD8 are both thought to have a role in colon cancer. They were both down-regulated in mild UC (Table 7.6). Nuclear factor of activated T cells acts through PTEN to regulate intestinal cell differentiation (Wang et al 2011). Its role in the colon is less clear. This was down-regulated in mild colitis in this study but not in severe colitis. NFATC1, down-regulated in severe colitis in this study, has been known to play a role in colon cancer genesis.

Table 7.6. Genes connected with Wnt-signalling whose mRNA expression was tested. "Not altered" indicates that expression was not statistically significantly altered in relation to the control biopsies. The "fold-down-regulation" was calculated as described in the text. Statistically significant values ($P<0.05$) as well as values showing trend to significance ($P<0.10$ & >0.05) are shown.

Gene	**Description**	**Group 1**	**Group 2**
FZD1	Frizzled homolog 1 (Drosophila)	Not altered	Not altered
FZD2	Frizzled homolog 2	Not altered	Not altered

	(Drosophila)		
FZD3	Frizzled homolog 3 (Drosophila)	Not altered	Not altered
FZD4	Frizzled homolog 4 (Drosophila)	Not altered	Not altered
FZD5	Frizzled homolog 5 (Drosophila)	Not altered	Not altered
FZD6	Frizzled homolog 6 (Drosophila)	Not altered	Not altered
FZD7	Frizzled homolog 7 (Drosophila)	2.64 fold-down-regulated, P=0.0875	Not altered
FZD8	Frizzled homolog 8 (Drosophila)	2.1 fold-down-regulated, P=0.0714	Not altered
FZD9	Frizzled homolog 9 (Drosophila)	Not altered	Not altered
LRP5	Low density lipoprotein receptor-related protein 5	Not altered	Not altered
LRP6	Low density lipoprotein receptor-related protein 6	Not altered	Not altered
VANGL2	Vang-like 2 (van gogh, Drosophila)	3.11 fold down-regulated, P=0.0542	Not altered
BCL9	B-cell CLL/lymphoma 9	Not altered	Not altered
BCL9L	B-cell CLL/lymphoma 9-like	Not altered	Not altered
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	Not altered	Not altered
LEF1	Lymphoid enhancer-binding factor 1	Not altered	Not altered
NFAT5	Nuclear factor of activated	Not altered	Not altered

	T-cells 5, tonicity-responsive		
NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	Not altered	6.11 fold down-regulated, P=0.0487
NFATC2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	Not altered	Not altered
NFATC3	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	Not altered	Not altered
NFATC4	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	4.50 fold down-regulated, P=0.0303	Not altered
PYGO2	Pygopus homolog 2 (Drosophila)	Not altered	Not altered
TCF7L1	Transcription factor 7-like 1 (T-cell specific, HMG-box)	Not altered	Not altered
TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)	Not altered	Not altered
TCF7	Transcription factor 7 (T-cell specific, HMG-box)	Not altered	Not altered

DISCUSSION

Ulcerative colitis is a disease of the colonic epithelium in which the epithelial cells of the colon ulcerate and slough off leaving ulcers in the colon that give rise to bleeding and diarrhoea. The nature of the primary defect in ulcerative colitis remains obscure, with current opinion favouring

the possibility that defects in barrier function as well as defects in innate immunity contribute to the genesis of the disease. However, this hypothesis remains to be proven.

Little or nothing is known about epithelial stem cell alterations in ulcerative colitis. The colonic crypts are shortened and deformed or branched and there is loss of surface epithelium. It is certain that there are alterations in epithelial stem cell function, signalling and renewal. The present studies identify some of the abnormalities of structure, function and distribution of epithelial stem cells in the colon in ulcerative colitis. Using the RNA-binding protein Musashi-1 as the primary locator of epithelial stem cells, the normal human colon has very few of these cells located at or close to the base of the colonic crypt. On the other hand, in patients with colitis, although the number is slightly lower than in normal colon, these cells are located away from the crypt base. Musashi-1 is an RNA binding protein that was originally discovered in neural cells as a homologue of the Drosophila protein. It is reported to have a central role in stem cell maintenance and self-renewal. It is used as a marker of stem cells in the colonic epithelium. The two direct targets of Musashi-1 are *numb* and *CDKN1A*. Binding of Musashi-1 to these mRNAs represses production of Numb protein and has effects on Notch and Hedgehog signalling (Sanchez-Diaz 2008). The Notch and Hedgehog pathways lead to Hes-1 downstream. Hes-1 is a DNA-binding protein that regulates transcription. In most tissues, Hes-1 and Notch work in the same direction, but in embryonal stem cells, Hes-1 appears to work in the opposite direction to Notch (Kobayashi 2010). In certain tissues, Hes-1 is downstream of wnt signalling and not Notch signalling (Kubo 2009). In most cell lineages, Hes-1 is thought to be important in protecting stem cells from premature senescence and inappropriate differentiation (Sang 2008). In the intestine, Hes-1 signaling leads to differentiation towards the columnar cell lineage. In the intestine, Hes-1 expression (mediated through Notch signalling) contributes to maintenance of

the proliferative crypt compartment. Loss of Notch signalling and Hes-1 leads to complete conversion of crypt progenitor cells to post-mitotic goblet cells (Riccio 2008). On the other hand, Math-1 signalling leads towards development of the secretory lineage (van Es 2010). This comprises of the goblet cells, the enteroendocrine cells (which require neurogenin-3 signalling in addition) and the Paneth cells (which require sox-9 signalling in addition).

In the studies described in this Chapter, Musashi-1 positive stem cells were located in the base of the colonic crypts in the normal colon, but abnormally located stem cells were seen in ulcerative colitis biopsies. The significance of this is not clear, but we can speculate that these changes reflect a response to colitis rather than being the primary cause of colitis. Numbers of these stem cells were lower in UC biopsies compared to control biopsies. Again it is not clear whether this change occurs in response to crypt injury and damage, or whether it is a primary cause. However, a gradient in Musashi-1 cell number was seen with normal colon having the highest number, and active colitis with the lowest number, while normal-appearing mucosa in ulcerative colitis patients showed intermediate numbers.

Hes-1 signalling appears to be quite active in most patients with UC compared to the controls, and is found in both epithelial cells in the crypts as well as lamina propria cells. Notch signalling leads to differentiation of intestinal stem cells into goblet cells (Van Es 2005). Activation of Hes-1 and Notch is therefore expected to lead to depletion of goblet cells, a characteristic histological feature of active ulcerative colitis.

The stem cell signalling pathway-focussed PCR array studies provided very interesting information. Two groups were compared to normal controls. Group 1 had chronic colitis but with low or absent inflammation in the mucosa, i.e. they had inactive disease under control with medication. Group had active disease with surface ulceration and moderate to severe

inflammatory infiltration in the lamina propria including presence of neutrophils in the crypt epithelium (cryptitis, crypt abscess). Expression of genes involved in maintaining multipotency, or in Sonic or Indian hedgehog signalling were not altered. Changes were found in genes that are involved in FGF-signalling, Notch-signalling, TGF-β-signalling, and Wnt signalling. In all cases, the relevant genes were down-regulated, and interestingly we did not find up-regulation of any of the genes. Down-regulation of FGFR1 in Group 2 indicates that there may be a deficiency in intestinal epithelial repair pathways in active inflammation in the colon. Down-regulation in Group 1 of caudal type homeobox 2 (CDX2) gene, which is involved in intestinal epithelial cell differentiation, may explain the crypt deformity and branching noted in ulcerative colitis. Notch2 and Notch3 were down-regulated in Group 1 indicating that these genes are specifically affected in ulcerative colitis. Notch2 is generally regarded as being found i lamina propria lymphocytes but may also be found in crypt epithelium. Not much is known of the role of Notch3 in the colon. Transforming growth factor-β is important in intestinal inflammation and repair. Specific defects in this pathway could reflect alterations in repair and inflammatory processes. The ACVRL1 gene, which encodes a cell surface receptor for TGF-β, was significantly down-regulated in Group 1 indicating a specific defect in ulcerative colitis unrelated to inflammation. SMAD3 and SMAD9 were down-regulated in both Groups 1 & 2. These genes are concerned with crypt proliferation and epithelial repair in the colon, and down-regulation of SMAD3 is known to accelerate epithelial repair processes in the colon (Furukawa et al 2011). Thus this may be a stem cell signalling event necessary to maintain the epithelial barrier in colitis. Genes involved in Wnt signalling were also altered in Group 1 – these included FZD7 and FZD8 which were down-regulated. Nuclear factor of activated T cells isotype 4 (NFATC4) was also down regulated in Group 1, while NFATC1 was down-regulated in Group 1.

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DISCUSSION

The gastrointestinal tract has one of the largest populations of epithelial stem cells in the body, which is responsible for the perpetual regeneration in this tissue. Initial studies using ^{3}H-thymidine labeling and autoradiography or electron microscopy demonstrated the presence and morphologic characteristics of these epithelial stem cells in the small intestine and colon, and a significant amount of the information regarding epithelial stem cells in the intestine derives from studies on the small intestine. The epithelial stem cells give rise to two populations of long-lived daughters, one committed to producing absorptive enterocytes, the other committed to producing secretory cell lineages (goblet, enteroendocrine, and Paneth cells). Paneth cells mature and remain in the crypt, interposed between a smaller population of epithelial stem cells, and produce antibacterial molecules such as cryptdins. Members of the three other epithelial lineages differentiate as they migrate out of crypts and move up the villi to a cellular extrusion zone located near the tip of each villus. The cycle of renewal is completed every 3-5 days. (De Santa Barbara et al 2003, Okamoto et al 2004, Stappenbeck et al 2003). The location, fate and signalling for epithelial stem cells in the colon is much less clearly delineated.

Certain clinical conditions are associated with severe disturbances of regeneration of the gastrointestinal epithelium. In the small intestine, these diseases include tropical sprue, radiation enteritis and mucositis secondary to chemotherapy. Inflammatory bowel disease is a more complex situation where there is ulceration of the epithelium as well as defects in the innate immune system of the gut. In many of these situations, the treatment is to attend to the underlying problem, such as the use of antibiotics in tropical sprue. There are several patients with refractory disease not responding to the specific therapy suggested above, where it may be useful to have novel therapies to regenerate intestinal epithelia (Bullard & Weaver 2002). Such situations could include radiation enteritis, inflammatory bowel disease and refractory sprue.

Unlike the situation with epithelial stem cells in the small intestine, the nature and signalling mechanisms of colonic epithelial stem cells are much less clearly understood. It has in fact been difficult to isolate these cells and show that they have the stem cell characteristics of asymmetric cell division and the ability to remain quiescent for long periods of time and begin to proliferate in response to appropriate signals. The present studies described in this thesis focused solely on colonic epithelial stem cells, with a view to understanding changes in stem cell numbers, location and signalling in colitis, and also to determine whether infusing proliferative colonic epithelial cells into the lumen in an experimental animal with extensively ulcerated colonic mucosa would allow transplantation of allogeneic epithelial stem cells.

Electron microscopic studies reveal that there is a degeneration of intestinal stem cells in specific disease entities including tropical sprue, radiation enteropathy, chemotherapy induced mucosal damage and one form of autoimmune enteropathy (Mathan et al 1975). These conditions, in particular, may benefit from replacement of stem cells. Occurrence of mutations in certain cells such as in patients with the APC (adenomatous polyposis coli) gene may lead to formation of polyps and eventually cancer. It is possible that replacement of these cells with stem cells containing the genetic characteristics to produce the normal protein will eventually become a form of therapy for patients with polyposis syndromes. Based on a number of premises, it has been suggested that stem cell transplantation may be useful in a variety of intestinal disorders including bile acid malabsorption and inflammatory bowel disease (Potten et al 2003, Stelzner et al 2003, Wirtz & Neurath 2003).

Traditionally a number of techniques have been used for separating villus from crypt cells in the intestine. Crypt cells are characterized by low levels of the brush border enzyme alkaline phosphatase, and by a high replicative ability shown by uptake of ^{3}H-thymidine. More

definitive isolation of these cells use fluorescence activated cell sorting or separation using magnetic bead technology. This is facilitated by the recognition of cell surface or cytoplasmic proteins unique to stem cells. Recently, the RNA-binding protein Musashi-1, and the Musashi-induced transcription factor Hes-1, have been described as possible markers of intestinal and colonic stem cells, as has beta-1-integrin (Kayahara et al 2003, Nishimura et al 2003, Fujimoto et al 2003). These proteins were therefore used to both identify stem cells and to characterize alterations in stem cell signalling in experimental colitis in animals as well as in human ulcerative colitis.

In these studies, we were able to isolate proliferative cells from the mouse colon epithelium, to maintain them in primary culture for over two weeks. Moreover these cells showed characteristics of epithelial stem cells including surface expression of beta-1-integrin, intracellular expression of Musashi-1, and the ability to form clones in a clonogenic assay. However, luminal infusion of these cells into the colon of mice with DSS colitis did not succeed in implanting these proliferative cells from a donor mouse in the ulcerated colon of the recipient mouse. We had expected that the ulcerated colon would express factors that would allow adherence of infused proliferative cells leading to their engrafting in a manner similar to engraftment of burns with donor skin or stem cells from skin. It is possible that this is an area that requires further study as this will be a good avenue to replace the epithelium in conditions characterised by extensive epithelial loss. There have been other stem cell therapy approaches to the treatment of inflammatory bowel disease (Singh et al 2010). Autologous haemotopoietic stem cell transplantation after immune conditioning has been effective in the treatment of refractory inflammatory bowel disease (Burt et al 2010). However, this treatment appears to work through a re-setting of the immune system rather than by replacing lost epithelial cells.

Similarly, mesenchymal stem cell transplantation has been beneficial in experimental colitis in mice induced either by trinitro benzene sulfonic acid or DSS (Hayashi et al 2008, Tanaka et al 2011) as well as in human inflammatory bowel disease (Liang et al 2011). In these animals, mesenchymal stem cells derived from bone marrow were injected into the serosa of the colon or injected intraperitoneally. Again, the mesenchymal stem cells do not appear to replace the ulcerated epithelium but rather they may act through release of immunomodulatory factors (Manieri & Stappenbeck 2011).

Epithelial stem cell populations were affected in human ulcerative colitis. Musashi-1-staining cells were found in the bases of colonic crypts in normal epithelium, while they migrated up to the sides of the crypt around the lower half in patients with ulcerative colitis. The number of these cells was also reduced in the ulcerated colonic mucosa in patients with ulcerative colitis. This is somewhat surprising since it would have been expected that stem cells numbers should in fact increase in patients with ulceration and loss of epithelium, as a response to this to help in repair. On the other hand, Hes-1 positive cells were not significantly altered in ulcerative colitis. In normal control subjects, the Hes-1 positive cells were abundantly found in the lower half of the crypts and they seemed to migrate upward in the crypt epithelium in ulcerative colitis patients. A large number of cells in the lamina propria also stained positive for Hes-1 indicating Notch signalling occurring in the lamina propria cells as well as in epithelium.

The stem cell signalling pathways in the colonic mucosa of ulcerative colitis patients were examined by pathway-focussed PCR arrays that determined the expression of 84 genes involved in stem cell signalling in various tissues. The expression of mRNA for these genes was compared with the expression in normal colonic biopsies obtained from patients with irritable bowel syndrome or rectal bleeding who did not have colonic mucosal abnormalities either by

colonoscopy or histology. Two groups of ulcerative patients were included – those with active disease which was moderate to severe histologically (indicating presence of surface ulceration and inflammatory infiltration in the lamina propria and neutrophils infiltration in the crypts) classified as Group 2, and those with chronic ulcerative colitis whose disease was in remission following adequate therapy (Group 1). We considered that the latter would show changes in stem cell signalling gene expression specific to the disease process in ulcerative colitis, while the former may show changes in stem cell signalling gene expression secondary to inflammation and ulceration. Using these groups we were able to discern the effects summarized in Table 8.1. Interestingly, there were relatively few changes in the group 2 with active UC where we would have expected more effects secondary to mucosal ulceration and inflammation. The specific effects noted in Group 1 suggest that genes involved in crypt homeostasis in the colon are specifically affected in chronic ulcerative colitis. It is very likely that these alterations reflect the effects of chronic disease. It is possible that they could be secondary to the use of medication used to control colitis, but in a small sample like this it is not possible to sort out the effect of medication on stem cell signalling in the colon. It is of interest that the pathways involved are all necessary for maintenance of normal epithelial cell morphology, differentiation and positioning in the crypts of the colon. NFATC4 and CDX2 are both involved in crypt cell differentiation and their down-regulation in UC in Group 1 may be responsible for the appearance of less differentiated crypt cells seen in some sections in biopsies from UC patients (for example Figure 7.6). Down-regulation of SMAD3 in both Group 1 & 2 probably reflect the need to switch on epithelial repair pathways in ulcerative colitis.

Table 8.1. Summary of changes in expression of genes related to stem cell signalling in colonic biopsies obtained from patients with ulcerative colitis (Group 1 inactive, Group 2 active) compared to normal control colon.

Gene group	**Group 1**	**Group 2**
Maintenance of multipotent stem cells	No change	No change
Hedgehog pathway genes	No change	No change
Fibroblast growth factor pathway genes	2.5 fold down-regulation of caudal type homeobox 2 (CDX2)	10.5 fold down-regulation of FGFR1
Notch pathway	2.1 fold down-regulation of Notch2 4.7 fold down-regulation of Notch3	No change
TGFβ Superfamily-pathway	3.8 fold down-regulation of Activin A receptor type II-like 1 5.5 fold down-regulation of SMAD3 3.4 fold down-regulation of SMAD9	22.0 fold down-regulation of SMAD3 4.7 fold down-regulation of SMAD9
Wnt pathway	2.6 fold down-regulation of Frizzled homolog 7 2.1 fold down-regulation of Frizzled homolog 8 3.1 fold down-regulation of Van Gogh like 2 4.5 fold down-regulation of Nuclear factor of activated T-cells, type 4	6.1 fold down-regulation of Nuclear factor of activated T-cells, type 1

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SUMMARY AND CONCLUSION

In **Summary**, these studies provide new insight into colonic epithelial stem cell physiology including alterations in ulcerative colitis and in experimental colitis induced by dextran sodium sulphate in mice. Furthermore, alterations in stem cell signalling in the colonic mucosa of ulcerative colitis patients are pinpointed most of which appear to be specific to the disease and only a few secondary to inflammation.

The specific **Conclusions** are as follows:

1. Expression of intracellular stem cell marker Musashi-1 and differentiation marker Hes-1 by immunocytochemistry and PCR revealed that proliferative cells are present in the pool of colonocytes isolated from mouse colon, and their distribution and numbers could be determined. In suspension cultures, we could identify the clones at the end of 14 days which were positive only for Musashi-1 and not Hes-1. So this confirms that zonal proliferative cells of the colon have the clonogenic potential by which it undergoes self-renewal by itself and forms progenitor cells.
2. Luminal infusion of proliferative colonic epithelial cells from donor mice into the colon of mice with dextran sodium sulphate colitis was not an effective method for epithelial stem cell transplantation from donor to recipient mice.
3. The comparison between proximal and distal colon of colonic epithelial stem cells represents that there was no difference observed in the numbers and distribution of these cells between proximal and distal colon of normal mouse as determined by immunostaining using the antibodies to Musashi-1 and Hes-1.
4. In mice with DSS colitis, there was no change in either proximal or distal colon in staining pattern against the antibodies Musashi-1 and Hes-1, even though the distal colon was more inflamed than the proximal colon.

5. There were significant changes observed between the colonic mucosal biopsies of control patients and colitis patients in both the numbers as well as in the location of stem cells in the colon. There was an increase in the numbers of stem cells in colitis patients when compared to control subjects and the stem cell location was also altered with the Musashi-positive cells being seen at the top of crypts in the colitis patients, while they were confined to the bottom of the crypts in normal controls.
6. There was no alteration in mRNA expression of genes related to stem cell multipotency, or in the Sonic and Indian Hedgehog pathways in ulcerative colitis.
7. mRNA expression of Fibroblast Growth Factor Receptor 1 was significantly down-regulated in active ulcerative colitis, while expression of Caudal type homeobox 2 gene was down-regulated in UC in remission. The former is involved in tissue repair and the latter in cell differentiation in the crypt.
8. Notch2 and Notch3 expression was down-regulated in inactive ulcerative colitis, reflecting alterations in Notch signalling pathways in the disease.
9. Alterations in transforming growth factor (TGF)-β signalling pathways were reflected as down-regulation of SMAD3 and SMAD9 expression in both active and inactive UC possibly reflecting a reparative response to injury in colitis. Down-regulation of ACVRL1 in inactive UC may be a more specific change in a cell surface receptor for TGF-β.
10. Wnt signalling pathways were affected in inactive UC, including down-regulatino of Frizzled 7 & 8 and nuclear factor of activated T cells 4 (NFATC4), reflecting altered regulation of genes relating to crypt epithelial cell differentiation in the colon. NFACT1 expression was down-regulated in active UC.

RECOMMENDATIONS

Gastrointestinal diseases are among the most common afflictions in the country. Ulcerative diseases of the gastrointestinal tract such as ulcerative colitis are on the rise due to unknown factors possibly related to changing lifestyles. Some patients have refractory forms of these conditions where the only treatment available may be surgical option of colectomy (if disease is limited to the colon) or the use of expensive medication such as infliximab. These studies identify specific defects in stem cells located in the crypts of the colon and alterations in the signalling pathways that could associate with these defects. The studies shed further light on the epithelial stem cell physiology in ulcerative colitis both in humans and in experimental animals. A better understanding of these disruptions will eventually allow the manipulation of these signals to enhance epithelial cell proliferation in ulcerative disorders of the intestine, to prevent the colon cancer that arises as a complication of ulcerative colitis, and to provide new tools in the therapeutic armamentarium to treat these distressing disorders.

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