STEM CELL IN GASTROINTESTINAL STRUCTURE AND NEOPLASTIC DEVELOPMENT

899

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Stem cells are primitive cells located in a specialised mesenchymal “niche” that lack expression of any definitive markers of lineage commitment and are therefore difficult to define and identify. Stem cells maintain their capacity for limitless self replication throughout the lifetime of their host, and can also divide to produce daughter cells, committed to the formation of every adult cell lineage within their tissue of origin. The stem cells of the gastrointestinal tract remain unidentified which has led to many conflicting hypotheses as to their precise nature and function. For example, the numbers and location of stem cells in the intestinal crypts and gastric glands have never been conclusively proven and, consequently, the clonal origins of these structures under normal circumstances and in neoplasia are clouded issues. The morphological events of gastrointestinal carcinoma formation are hotly debated, with two main conflicting hypotheses of the mechanisms of expansion of a mutated stem cell clone. However, with the emergence of the molecular pathways governing gastrointestinal stem cell function, and the identification of putative intestinal molecular stem cell markers, such as Musashi-1, comes a clearer insight into the properties of the gastrointestinal stem cell. Adult stem cells from several tissues can leave their niche and engraft into extraneous tissues, including the gastrointestinal mucosa and underlying mesenchyme, and transform to produce adult cell lineages common to these foreign environments. This process is optimal when the requirement for regeneration is enhanced (that is, in diseased or damaged tissue) and indeed, the contribution of transplanted bone marrow stem cells to intestinal myofibroblasts is significantly upregulated in colitis. However, adult stem cell plasticity has recently been disparaged by reports suggesting that stem cells spontaneously fuse with indigenous adult cells to form a diploid cell with an aberrant karyotype, and it is important to investigate if bone marrow cells contribute to a gastrointestinal stem cell population, and indeed the mechanisms by which they do so. Identification of the origins, location, and molecular regulators of the gastrointestinal stem cell will provide a clearer understanding of normal gastrointestinal function and the genetic pathways involved in neoplastic change.

INTRODUCTION

Stem cells are large, primitive cells that do not express markers of lineage differentiation, and are consequently difficult to define and characterise morphologically. Fundamentally, stem cells provide the foundations of every organ in the body, with their ability for self renewal by symmetrical division and their capacity to generate the entire adult cell component within their indigenous tissue via asymmetrical cell division. Stem cells regulate the rate of cell production in a tissue to maintain homeostasis, and can upregulate cell turnover concurrent to increasing regenerative demand dictated by damage or disease. Gastrointestinal stem cells undergo multipotent division to produce the entire specialised cell repertoire of the gastrointestinal tract. The rapid turnover rate of committed cells in this tissue means a homeostatic balance must be maintained by stem cells in their regulation of cell proliferation, senescence, and apoptosis. Following the opportune discovery that adult stem cells are not restricted to cell production within their native organ, but can engraft within extraneous tissues and produce specialised adult cell lineages within this new environment, the potential of adult stem cells in regenerative biology, and indeed in clinical medicine, has become apparent. Adult stem cells of several tissues have been shown to form adult cell lineages within foreign tissues, a phenomenon termed “plasticity” or “transdifferentiation”. For example, transplanted adult bone marrow stem cells (BMSC) contribute to several non-haematopoietic adult cell types, including epithelial and mesenchymal lineages in the mouse and human gastrointestinal tract.^{1–3} It is, however, important to demonstrate that these BMSC-derived lineages are functional, with a clonogenic capacity, and not merely a transdifferentiated phenotype. This has been demonstrated in the fumarylacetoacetate hydrolase (FAH^{-/-}) deficient mouse, which develops a fatal metabolic liver disease resembling human type I hereditary tyrosinaemia, and can be rescued by transplantation of

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haematopoietic BMSC, which migrate to the liver and transform into FAH synthesising hepatocytes.⁶ Although bone marrow cells have been shown to contribute to multiple cell lineages in the gastrointestinal tract, their capacity for functional tissue regeneration and clonal expansion is currently not known. Further investigation into the mechanisms of adult stem cell transdifferentiation, and indeed the origins of both epithelial and mesenchymal lineages in the gastrointestinal tract, may provide a clearer insight into the cellular mechanisms of normal gastrointestinal function, and the genetic and cellular pathways leading to the onset of disease and neoplasia.

Gastrointestinal epithelial cell lineages

There are four principal epithelial cell lineages in the gastrointestinal tract, each displaying variations in morphology and function in relation to their location. “Columnar” cells, termed “enterocytes”, in the small intestine and “colonocytes” in the large intestine, comprise the principal epithelial cell lineage of the gastrointestinal mucosa; “mucin secreting” cells, known as goblet cells in the small intestine and colon, and gastric foveolar mucous cells in the stomach; “endocrine”, “neuroendocrine”, or “enteroendocrine” cells which function in peptide hormone secretion; and “Paneth” cells in the small intestine and ascending colon, which contain large apical secretory granules and express specific proteins, including lysozyme, tumour necrosis factor, and the anti-bacterial cryptin molecules. Other less common cell lineages are also present, such as caveolated cells and M (membranous or microfold) cells. In the stomach, the peptic/chief or zygomatic cells are located at the base of the glands in the fundic and body regions, and acid secreting parietal (oxyntic) cells are found in the body of the stomach in the base of the glands (reviewed by Wright⁷). The epithelial cell lineages of the gastrointestinal tract undergo constant turnover, with complete self renewal every 2–7 days under normal circumstances, which increases following tissue damage. This complex hierarchical arrangement of proliferating and

differentiated cells in the gastrointestinal tract is regulated by the multipotent gastrointestinal stem cells which, despite extensive scrutiny, remain unidentified due to their highly primitive nature and resultant lack of any definitive phenotypic and morphological markers. The exact numbers and location of the gastrointestinal stem cells is the topic of considerable debate although they are believed to maintain a polarised epithelial hierarchy due to their location at the origin of cell flux in the gastric glands and intestinal crypts. In the small intestine they are believed to be located in the crypt base just superior to the Paneth cells, and in the large intestine they are thought to be located in the mid crypt of the ascending colon and in the crypt base of the descending colon.⁷ In the stomach, migration of differentiating cells in the gastric glands is bidirectional from the neck/isthmus region in the centre of the gland, which is therefore assumed to be the location of the stem cell niche.⁸

Clonal origins of the gastrointestinal epithelium

The early “unitarian hypothesis” states that the epithelial cell lineages of the gastrointestinal tract are clonal populations derived from a single stem cell,^{9,10} although opposing reports of between 4 and 6 stem cells in intestinal crypts¹¹ and stem cell numbers of up to 16 or more in a single crypt have also been suggested.¹² Stem cell number may fluctuate throughout the crypt cycle, with a threshold number of stem cells being the signal for crypt replication, or “crypt fission”, to occur,¹³ and it is also postulated that stem cell number varies throughout different regions of the gastrointestinal tract.¹⁴ Functionally, the gastrointestinal stem cell can be demonstrated by its ability to regenerate entire intestinal crypts and villi subsequent to irradiation damage, which is only possible if a stem cell maintains its viability within the niche.¹⁵ Monoclonal intestinal crypts have been demonstrated following irradiation, showing that a single multipotent surviving stem cell can regenerate an entire crypt, thus substantiating the unitarian hypothesis, albeit in damaged mucosa.¹⁶ Tissue clonality studies are often undertaken in chimeric and mosaic

Figure 1 Clonal origin of normal human intestinal crypts and small intestinal villi. (A) Monoclonal origin of human colonic crypts: normal colonic mucosa in an XO/XY mosaic individual stained by in situ hybridisation for a Y chromosome specific probe showing a XO crypt (central) surrounded by two XY crypts (courtesy of M Novelli). (B) Villi, receiving cells from more than one crypt of different clonal derivation, show a polyclonal pattern in this XO/XY patient. Apart from the occasional Y chromosome positive inflammatory cell (red chromosome label) the majority of cells on the right of this villus are XO (green chromosome paint) whereas on the left side the cells are XY (red and green chromosome paint) (courtesy of R Poulson).

animals where the divergent founder germline cell populations are identified by their unique phenotypic markers. For example, in the XX/XY chimeric mouse, male and female derived cells are distinguished by their expression, or lack of, a Y chromosome,¹⁷ and in C57BL/6J Lac (B6)' SWR mouse embryo aggregation chimaeras the lectin *Dolichos biflorus agglutinin* binds to sites on the B6 derived but not SWR derived cells.¹⁰ In both models, clonality studies have indicated that gastric glands¹⁷ and intestinal crypts are monoclonal structures.¹⁰ Female mice, heterozygous for a X linked polymorphism causing reduced expression of glucose-6-phosphate dehydrogenase (G6PD), are naturally mosaic and allow in situ analyses of the clonal architecture of the cells of the gastrointestinal tract. In the small intestine of these mice, crypts are monoclonal structures although villi are of polyclonal derivation, thought to be formed from the migratory differentiated progeny of more than one monoclonal intestinal crypt.¹⁸ In humans, the clonal origins of intestinal crypts and villi appear analogous to that in the mouse. For example, in a rare XO/XY patient with familial adenomatous polyposis (FAP), intestinal crypts were monoclonal populations as the entire epithelial cell component of each crypt studied either expressed, or lacked, a Y chromosome (fig 1A). In the small intestine of this patient, the villus epithelium contained a mixture of XO and XY cells, and were therefore polyclonal structures, believed to be composed of cells migrating from multiple crypts (fig 1B).¹⁹ It is important to note that where tumours are concerned, clonality studies are not strictly sustainable if they overlook the vital consideration of "patch size", wherein a "patch" is described as the number of cells of a single genotype within an area of tissue, derived from either a single clone or from the coalescence of multiple clones of the same lineage.^{20–21} Clonality must be determined at the patch edge, as it is not possible to decipher if cells within the centre of a patch are truly clonal or simply monophenotypic, formed from several stem cells of the same genotype. Heterozygosity for the G6PD Mediterranean mutation (563 C→T) is present in 17% of Sardinian females, permitting analyses of patch size by G6PD immunohistochemical staining. Of 10 538 colonic crypts analysed from nine patients carrying the G6PD Mediterranean mutation, patch size in the colon was observed to be relatively large, containing up to 450 crypts. No evidence of any crypts with a mixed phenotype was observed in 2260 crypts located at the periphery of a patch, indicating that colonic crypts are indeed monoclonally derived, consistent with results obtained previously²² (fig 2).

Recent DNA labelling studies have provided an insight into the mechanisms of stem cell proliferation and genome protection in the mouse small intestine. By labelling DNA template strands of intestinal stem cells with tritiated thymidine (HTdR) during development or in tissue regeneration, and by bromodeoxyuridine (BrdUrd) labelling the newly synthesised daughter strands, segregation of the two DNA strands in cell division can be studied. The original template DNA is retained within the stem cell although newly synthesised BrdUrd labelled strands are passed on to the daughter cells that are committed to leave the stem cell niche and differentiate. This study demonstrates that stem cells undergo asymmetrical division, and that by discarding the newly synthesised DNA, which is more prone to replication induced mutation, the stem cell utilises an innate mechanism of genome protection.²³

Figure 2 Demonstration of patch size is vital in studies to determine clonal origin of intestinal crypts. Glucose-6-phosphate dehydrogenase staining pattern in cross section of crypts within normal colonic mucosa showing large patches of crypts with irregular patch borders (from Novelli and colleagues²² with permission).

The gastrointestinal epithelial stem cell niche

It is generally accepted that stem cells in most tissues reside within a so-called stem cell compartment, or "niche", which provides and maintains an optimal microenvironment for stem cell function. The gastrointestinal epithelial stem cell niche is believed to be formed and maintained by the underlying cells of the mesenchymal lamina propria and their secreted basement membrane factors, which regulate stem cell behaviour through the paracrine secretion of various growth factors and cytokines, the receptors for which are situated on gastrointestinal epithelial cells (reviewed by Powell and colleagues²⁴). The "conceptual" stem cell niche should possess three standard constituents: the supporting cells and their secreted extracellular matrices to regulate stem cell behaviour, the target cell range covered by the signalling molecules, and the stem cells themselves.²⁵ Functionally, a niche is characterised by its persistence on removal of stem cells, and conversely, if stem cells are extracted from their niche they cease to retain their stem cell potential or "stemness".²⁶

Molecular markers of gastrointestinal epithelial stem cells

Musashi-1 (Msi-1) is a neural RNA binding protein, the mammalian homologue of a *Drosophila* protein, required for asymmetric division of sensory neural precursor cells^{27–28} and recently demonstrated to be highly expressed in mammalian neural stem cells.²⁹ The transcriptional repressor Hes-1 is essential for neural stem cell self renewal and suppresses neural stem cell differentiation into neuronal lineages^{30–32} and is a downstream target in the Notch signalling pathway of cellular differentiation,³³ described later in this review. Moreover, Msi-1 has recently been demonstrated to positively regulate Hes-1 transcription, suggesting a close interaction between Msi-1 and Hes-1.³⁴ Msi-1 and Hes-1 proteins are coexpressed in cells just superior to Paneth cells in the small intestine, the postulated stem cell region. Hes-1 has a more widespread expression than Msi-1 and is also expressed, albeit in reduced levels, in epithelial cells migrating towards the villus tip. It is suggested that colocalisation of Msi-1 and

Hes-1 in cells located just above Paneth cells is indicative of the stem cell population in the mouse small intestine, and that Hes-1 expression alone represents proliferating cells committed to differentiation that have migrated outwith the stem cell niche.³⁵ Musashi-1 mRNA and protein expression has also been confirmed in putative stem cells in neonatal and adult intestinal crypts in mice,²³ and has recently been demonstrated in the human colon in epithelial cells located between positions 1 and 10 in crypts (fig 3).³⁶ These studies implicate Musashi-1 as a possible gastrointestinal stem cell marker.

BONE MARROW STEM CELL PLASTICITY IN THE GASTROINTESTINAL TRACT

Studies of stem cell plasticity have shown that the differentiation pathways of adult cell lineages in the gastrointestinal tract may be less restricted than previously assumed. Transplanted BMSC have been shown to transdifferentiate to form both epithelial and mesenchymal cell

Figure 3 Musashi-1 expression within human colon crypts—a putative stem cell marker. Confocal imaging of human colon crypts (red: propidium iodide; green: Musashi-1). Several Musashi-1 positive cells were found near the base of the crypts (from Nishimura and colleagues³⁶ with permission).

lineages in both the mouse and human gastrointestinal tract. Lethally irradiated female mice injected with a single male haematopoietic BMSC demonstrated donor derived epithelial cells in the oesophagus, stomach, small intestine, and colon, 11 months after transplantation.³ Similarly, in patients who received a peripheral blood stem cell transplant, epithelial cells of donor origin were observed throughout the gastrointestinal mucosa.⁴ A purified mesenchymal stem cell population injected into early mouse blastocysts was shown to engraft within the gastrointestinal tract and transdifferentiate to form intestinal epithelial cells,⁵ and in the small intestines and colons of lethally irradiated female mice that were rescued by a BMSC transplant from male mouse donors, and in gastrointestinal biopsies from female patients who suffered graft versus host disease following bone marrow transplant from a male donor, transplanted BMSC were shown to contribute to a population of myofibroblast cells, the intestinal subepithelial myofibroblasts (ISEMF). These cells are located in the lamina propria subjacent to the epithelial mucosa and regulate intestinal epithelial cell function via epithelial:mesenchymal interactions.²⁴ Transplanted BMSC contribute to ISEMFs in both the mouse and human small intestine and colon. This was observed as early as one week post-transplant in the mouse small intestine and colon, and by six weeks post-transplant almost 60% of all ISEMFs in the mouse colon were of donor origin.² To investigate the functional capacity of these donor derived ISEMFs, a murine model of experimental colitis with similarities to Crohn's disease in humans was used to determine the response of BMSC to regenerative stress. Colitis was induced six weeks after a sex mismatched BMSC transplantation, and results showed that transplanted BMSC significantly increase their contribution to ISEMFs in the lamina propria in colitis compared with normal colons, with 76.57% of ISEMFs derived from the transplanted stem cells in severe disease. In both normal and diseased colons, donor derived ISEMFs were frequently present as cellular columns spanning from the base of the crypt to the intestinal lumen (fig 4), suggesting that they proliferate from a common progenitor BMSC derived ISEMF, although further investigation of the proliferative status of these cells is required. Transplanted BMSC also transdifferentiate to form fibroblasts and smooth muscle cells in the lamina propria and mucosal layers, indicating that transplanted adult BMSC contribute to multiple specialised adult gastrointestinal mesenchymal lineages in diseased tissue. Additionally, perivascular smooth muscle cells and endothelial cell lineages derived from transplanted cells were observed in newly formed blood vessels in inflamed colons, outlining a potential role of transplanted BMSC in vasculogenesis. As bone marrow derived epithelial cells appear as single, apparently random, entities, it does not appear that bone marrow cells engraft within the gastrointestinal epithelial cell niche and contribute to stem cells with a capacity for clonal expansion. However, the frequent appearance of columns of bone marrow derived myofibroblasts is suggestive of clonal expansion from a progenitor stem cell and it is possible that bone marrow can contribute to a myofibroblast stem cell, which highlights the potential for bone marrow cells as regulators of epithelial cell function via mesenchymal:epithelial paracrine interactions. These results substantiate the importance of the adult BMSC in the treatment of human diseases, such as inflammatory bowel disease.¹

Figure 4 Bone marrow derived intestinal subepithelial myofibroblasts (ISEMF) are present in cellular columns in the lamina propria of the mouse colon, reaching from the base of the crypt to the intestinal lumen. Female mouse colon six weeks following a bone marrow transplant from a male mouse donor. Bone marrow derived ISEMFs are present as Y chromosome positive cells, immunoreactive for α -smooth muscle actin (SMA). The Y chromosome is seen as a brown/black punctate density, and red cytoplasmic staining indicates immunoreactivity for α -SMA (courtesy of M Brittan).

Mechanisms of bone marrow stem cell plasticity: fusion or transdifferentiation?

Recent doubt has been cast on the validity of the mechanisms of stem cell plasticity, with reports inferring that stem cells merely fuse with indigenous cells within a tissue to form a diploid hybrid cell, rather than entering a foreign tissue and crossing lineage borders to transform into a divergent cell type. In cocultures of embryonic stem (ES) cells, with either GFP expressing neural stem cells³⁷ or GFP expressing BMSC,³⁸ adult stem cells appeared to fuse with ES cells when extracted from their niche environment. However, these studies observed genetically modified cells in culture, and are not directly analogous to *in vivo* observations of adult stem cell plasticity. Furthermore, these reported fusion events were too infrequent to account for reported BMSC plasticity *in vivo*

in some tissues (2–11 hybrid clones were formed per 10^6 BMSC³⁸), as six weeks following BMSC transplant almost 60% of intestinal subepithelial myofibroblasts in the normal mouse colon were derived from transplanted cells,² and likewise, transplanted BMSC contribute up to 13% of epithelial cells in human gastrointestinal mucosa.³⁹ To further refute the conjecture that adult stem cells undergo spontaneous fusion with adult cells, female patients transplanted with mobilised peripheral blood stem cells from male donors expressed a normal component of X and Y chromosomes in donor derived gastrointestinal epithelial cells,⁴ and bone marrow derived cells have been identified in the pancreatic islets of Langerhans, functionally typical of endocrine beta cells, with no evidence of cell fusion.⁴⁰ However, recent evidence has emerged to support the fusion hypothesis in the mouse liver, as the FAH^{-/-} mouse, mentioned previously, develops a fatal hereditary tyrosinaemia type 1 and can be rescued by transplantation of purified hepatic stellate cells (HSCs) which form functional hepatocytes and regenerate the damaged organ. Although initial engraftment of the damaged liver by transplanted HSCs is low (one donor derived hepatocyte per one million indigenous hepatocytes), donor derived hepatocytes undergo clonal expansion to repopulate the liver. Cytogenetic analyses of donor derived hepatocytes in repopulated livers showed that most, if not all, nuclei expressed a karyotype indicative that fusion had occurred between the transplanted donor HSC and host hepatocyte, to form a heterokaryon, albeit with a hepatocyte specific phenotype.^{41–42} It is possible that, as hepatocytes are frequently polyploid and thus carry multiple sets of chromosomes, the liver presents a unique environment for the formation of fused hybrid cells. Similar heterokaryon formation by a transplanted bone marrow cell with cardiomyocytes,⁴³ and Purkinje cells,^{44–45} have subsequently been demonstrated. The underlying principle, that transplanted BMSC rescue an otherwise fatal metabolic disease, is maintained and should not be overlooked, although it is irrefutable that the mechanisms by which adult stem cells provide this therapy require further investigation.

PATHWAYS OF CELLULAR DIFFERENTIATION IN THE GASTROINTESTINAL TRACT

An improved understanding of the molecular pathways that regulate proliferation and differentiation of cells in the gastrointestinal tract will provide a clearer insight into the location and behaviour of the gastrointestinal stem cell(s). An increasing number of genes and their ligands and receptors are being identified that are expressed by epithelial and mesenchymal cells in the gastrointestinal tract and are involved in the regulatory molecular pathways of epithelial cell proliferation and differentiation in both the normal and neoplastic gastrointestinal mucosa.

The Wnt/ β -catenin signalling pathway

In the canonical Wnt pathway, APC (adenomatous polyposis coli gene) forms a subcellular trimeric complex with axin and glycogen synthase kinase 3β (GSK3 β), resulting in phosphorylation, ubiquitination, and proteosomal degradation of β -catenin, and thereby maintaining low levels of cytosolic and nuclear β -catenin by this tumour suppressor complex. The signalling protein, Wnt, of which there are 19 family members identified in humans, activates the cytoplasmic phosphoprotein “dishevelled” through its receptor “frizzled”, causing inhibition of GSK3 β and a resultant accumulation of

cytosolic β -catenin.⁴⁶ β -Catenin then translocates to the nucleus and interacts with members of the Tcf/LEF (T cell factor/lymphocyte enhancer factor) family of DNA binding proteins, converting them from transcriptional repressors to activators and hence, causing activation of downstream target genes which increase cellular proliferation (fig 5), including c-myc, tcf-1, cyclin D1, c-Jun, Fra-1, urokinase-type plasminogen activator receptor, fibronectin, CD44, and the matrix metalloproteinase *matrilysin*, many of which have carcinogenic potential in the gastrointestinal tract (reviewed by Polakis⁴⁷ and Bienz and Clevers⁴⁸). When the Wnt signal is removed, APC extracts β -catenin from the nucleus, and the transcriptional repressor function of Tcf is restored.⁴⁹ The Wnt/ β -catenin pathway plays a key role in malignant transformation, as 85% of human sporadic colorectal tumours are reported to have an *APC* mutation.⁴⁶ This *APC* mutation renders the GSK3 β /Axin/APC complex incapable of destabilising β -catenin, thereby leading to an accumulation of nuclear β -catenin/Tcf/LEF complexes, and a subsequent increase in target gene transcription and cell proliferation which can lead to tumour formation.^{50 51}

The Tcf/LEF DNA binding protein family

There are four known members of this family of transcription factors: Tcf-1, LEF1, Tcf-3, and Tcf-4. Tcf-4 is expressed in high levels in the developing intestine from E13.5 and in the epithelium of the adult small intestine and colon and in colon carcinomas. In colonic epithelial cells in patients with a loss of function of the *APC* or β -catenin genes, nuclear accumulation of β -catenin/Tcf-4/LEF complexes implicates Tcf-4 in the ensuing uncontrolled target gene transcription, and subsequent upregulation of cell proliferation that can often lead to tumorigenesis.⁵³ In the absence of β -catenin, the Tcf/LEF family recruit the corepressor proteins "Groucho" and CREB binding protein to the downstream Wnt target genes and

inhibit their transcription (reviewed by Barker and colleagues⁵⁴). The Tcf-4 knockout mouse is devoid of proliferating cells in the small intestine, and is presumed to lack a functional stem cell compartment. It is thereby proposed that Tcf-4 is essential in establishing the stem cell population within the niche of the small intestinal crypts, and is believed to be activated by a *Wnt* signal from the underlying mesenchymal cells which form the stem cell niche.⁵⁵

Cdx-1 and Cdx-2 homeobox genes

The mammalian homeobox proteins Cdx-1 and Cdx-2 also appear to play an important role in intestinal epithelial stem cell transcriptional regulation, with a particular influence on gastrointestinal metaplasia. Cdx-1 is expressed throughout the proliferative compartment of the developing and adult mouse intestinal crypt epithelium,⁵⁶ and both Cdx-1 and Cdx-2 mRNAs show restricted expression in the epithelial mucosa of the human small intestine and colon.^{57 58} The Tcf-4 knockout mouse, mentioned above, does not express Cdx-1 in the small intestinal epithelium, implying that Cdx-1 is a direct downstream target of the Tcf-4/ β -catenin complex in the Wnt signalling pathway and is employed in the development of the epithelial stem cell niche.⁵⁹ Expression of Cdx-1 is reduced in proliferating epithelial cell nuclei in colonic crypts concurrent with their progression to adenomas and adenocarcinomas,⁵⁹ although as no colonic tumours develop in the Cdx-1 null mouse this molecule does not appear to have direct tumour suppressing properties.⁵⁶ Cdx-2 is expressed in all epithelial cell nuclei in the upper regions of the crypts of the descending colon to the rectum, with decreasing expression parallel to an increasing degree of dysplasia in these cells.⁶⁰ Region specific genes such as Cdx-1, Cdx-2, and Tcf-4 appear to define the morphological features of the specialised regions of the intestinal epithelium and regulate stem cell proliferation and differentiation.

Figure 5 The Wnt signalling pathway. (A) In the absence of Wnt signalling, Dishevelled is inactive (Dsh_i) and Drosophila Zeste-white 3 or its mammalian homologue glycogen synthase kinase 3 (Zw3/GSK3) is active. β -Catenin (black dumbbell), via association with the APC-Zw3/GSK3 complex, undergoes phosphorylation and degradation by the ubiquitin-proteasome pathway. Meanwhile, T cell factor (Tcf) is bound to its DNA binding site in the nucleus where it represses expression of genes such as Siamese in *Xenopus*. (B) In the presence of a Wnt signal, Dishevelled is activated (Dsh_a) leading to inactivation of Zw3/GSK3 by an unknown mechanism. β -Catenin fails to be phosphorylated and is no longer targeted into the ubiquitin-proteasome pathway, instead it accumulates in the cytoplasm and enters the nucleus where it interacts with Tcf to alleviate repression of the downstream genes and provide a transcriptional activation domain (from Willert and Nusse⁵² with permission).

Figure 6 The Math1 signalling pathway. (A) Low power section of adult mouse small intestine. Precursor cells (brown) are stained for cyclin PCNA (proliferating cell nuclear antigen); enterocytes (red) express intestinal alkaline phosphatase; goblet cells (blue) secrete mucins. The inset shows a high power image of small intestinal enterocytes and goblet cells. (B) Math1, a component of the Notch signalling pathway, influences intestinal epithelial cell fate decisions. In crypt progenitor stem cells that express high levels of Notch, the Hes1 transcription factor is switched on and expression of Math1 and of other "prosecretory" genes is blocked. The result is that the precursor cells become enterocytes. In cells expressing low amounts of Notch, levels of Delta are high, production of Hes1 is blocked, and Math1 expression is induced. Production of the Math1 helix-loop-helix transcription factor allows precursor cells to make a choice: whether to become goblet cells, Paneth cells, or enteroendocrine cells (Vi, villus; Cr, crypt) (A and B from van Den Brink and colleagues⁶⁸ with permission). (C) Math1 is essential for secretory cells. Whether Math1 expressing cells descend directly from stem cells or an intermediate progenitor remains unknown. Sec, secretin; L, glucagon/peptide YY; CCK, cholecystokinin; SP, substance P; 5HT, serotonin; Som, somatostatin; GIP, gastric inhibitory peptide; Gas, gastrin (from Yang and colleagues⁶⁷ with permission).

The forkhead family of transcription factors

The forkhead, or winged helix, family of transcription factors, of which there are nine members identified in mice, produce the Fox (forkhead box) proteins.⁶¹ Mice with a heterozygous targeted mutation of the forkhead homologue 6 (fkh-6), or Fox1 gene, which is ordinarily expressed by gastrointestinal mesenchymal cells, display an atypical gastrointestinal epithelium with branched and elongated glands in the stomach, elongated villi, hyperproliferative crypts, and goblet cell hyperplasia due to increased epithelial cell proliferation. These Fox1 mutants have increased levels of heparin sulphate proteoglycans (HSPGs), causing overactivation of the Wnt/ β -catenin pathway and a subsequent increase in target cell proliferation, thereby demonstrating an indirect regulation of the Wnt/ β -catenin pathway by Fox1 mediated HSPG production.⁶²

TGF- β and Smad signalling pathway

The transforming growth factor β (TGF- β) family are known inhibitors of gastrointestinal epithelial cell proliferation. Under normal circumstances, TGF- β forms a multimeric complex with the serine-threonine TGF- β type I (T β R1) and type II (T β R2) receptors, causing phosphorylation of Smad2 and Smad3 cytoplasmic proteins and their formation of a heteromeric complex with Smad4. This complex then translocates to the nucleus where it interacts with transcriptional activators and coactivators to generate TGF- β target gene transcription.^{63–64} Disruption of this TGF- β /Smad signalling pathway causes upregulation of epithelial cell proliferation, and can lead to tumorigenesis, and Smad2 and Smad4 are frequently inactivated in human cancers confirming their function as tumour suppressor genes.⁶⁵ Mice with heterozygous targeted mutations in the Smad4 and APC genes develop adenomatous polyps in the small intestine and colon

due to loss of heterozygosity (LOH) of the APC and Smad4 wild-type alleles. These lesions progress to form adenocarcinomas with an increased malignant nature than those formed in mice with a heterozygous mutation of the APC allele only.⁶⁶ This model implies a reciprocal interaction between the TGF- β and Wnt signalling pathways in the progression of intestinal carcinogenesis, wherein LOH of genes from both pathways is required before malignant transformation can occur.⁶⁶

Regulation of stem cell fate and patterning in gastrointestinal crypts and villi

The Notch signalling pathway regulates gastrointestinal epithelial cell fate and differentiation of the four specialised epithelial lineages of the gastrointestinal tract. This pathway supports the unitarian hypothesis that a single stem cell gives rise to all mature intestinal epithelial cell lineages. Increased levels of Notch protein negatively regulate the transcription of the Math1 gene, a basic loop-helix transcription factor, via upregulation of the Hes1 transcriptional repressor. Mice with targeted deletion of the Math1 gene fail to develop goblet, Paneth, and enteroendocrine cell lineages in the small intestine, and these Math1 negative epithelial cell progenitors solely form enterocytes.⁶⁷ Conversely, reduced Notch expression and subsequent accumulation of its ligand, Delta, increases Math1 expression by blocking Hes1, causing cells to transdifferentiate to form goblet, Paneth, and enteroendocrine lineages in the small intestine (fig 6).⁶⁸ Hes1 knockout mice display elevated Math1 expression with a concurrent increase in the numbers of goblet, Paneth, and enteroendocrine cells and a reduced enterocyte population.⁶⁹

The E2F family of transcription factors regulate cell proliferation, and E2F4 is expressed in epithelial cells within the proliferative regions of crypts in the embryonic and adult

mouse small intestine and colon. The E2F4 knockout mouse fails to develop intestinal crypts and displays a thickened lamina propria, indicative of the requirement of E2F4 in the development of the proliferative compartment of the intestinal crypts, although little is known of the mechanisms and pathways which control E2F4 during development.⁷⁰

The sonic hedgehog pathway of gastric epithelial cell differentiation

The sonic hedgehog (Shh) gene encodes a morphogenetic signalling protein with an important regulatory role in gastrointestinal development, with an emphasis on gastric gland formation.⁷¹ In the gastric glands, epithelial cell differentiation is believed to occur bidirectionally from a stem cell(s) located in the central isthmus/neck region,⁸ although the molecular events surrounding this polarised cell proliferation and differentiation are unclear. The high level of Shh expression by gastric epithelial cells in the stomach of both adult mice and humans has led to proposals that Shh promotes gland cell differentiation with negative regulation of progenitor cell and gland cell proliferation.^{72–73} Mice with a targeted homozygous deletion of Shh fail to develop gastric epithelium and display strikingly similar defects to those observed in the Fox1 mutant mice mentioned above.⁷⁴ These mice lack Fox1 expression, and thus Fox1 is thought to be a downstream target of the Shh signalling pathway, verified by its inhibition by bone morphogenetic protein 4 (BMP4), a member of the TGF- β superfamily.⁷⁴ By blocking Shh signalling in mice with cyclopamine, there is a marked increase in gastric gland cell proliferation and levels of expression of the Shh receptor “patched” (Ptc), and three known transcriptional targets of Shh: transcription factor HNF3 β (Fox2a), BMP4, and islet factor-1 (Isl-1) are decreased.⁷² In the mouse, Shh, Ptc, HNF3 β , and BMP2 are expressed within gastric epithelial cells⁷² whereas Fox1 and BMP4 are expressed in myofibroblasts,^{72–74} providing further evidence of epithelial:mesenchymal interactions in the regulation of gastrointestinal cell proliferation and differentiation. The observed negative regulation of epithelial cell proliferation by Shh was unexpected as Shh generally increases cell proliferation.⁷⁵ However, bone morphogenetic proteins are known inhibitors of cell proliferation, and the observed increase in proliferation when Shh signalling is blocked with cyclopamine, could result from downstream inhibition of BMP4. The GATA family of transcription factors are important in development of many endodermal structures in a variety of organisms. GATA-4 is vital for gastric epithelial morphogenesis, as mice with a targeted homozygous deletion of the *Gata4* gene show defects in gastric gland morphogenesis and fail to form terminally differentiated adult epithelial gastric lineages. The increased expression of Shh in gastric epithelial cells of these mice indicates that GATA-4 interacts either directly or indirectly with Shh to regulate epithelial cell proliferation.⁷⁶

THE GASTROINTESTINAL STEM CELL IN INTESTINAL NEOPLASIA

Onset of colorectal neoplasia

The gastrointestinal tract is one of the most frequent sites of carcinogenesis due to its continual self renewal and the resultant large numbers of daily mitotic events in this tissue. Gastrointestinal epithelial cells migrate upwards within the intestinal crypts and villi and are shed into the intestinal lumen, and consequently the lifespan of a gastrointestinal

epithelial cell is shorter than the length of time taken to genetically induce neoplastic change in this cell. This implicates the perpetual gastrointestinal stem cell as the target for genetic alterations, and consistent with previous investigations indicating that intestinal crypts are monoclonal structures, the resultant lesions may also be predicted to be monoclonal in origin. However, conflicting data have emerged from such studies, and the pathways and mechanisms of gastrointestinal neoplasia are as yet unresolved. To investigate the role of the stem cell in gastrointestinal neoplasia, we can use the widely studied adenoma:carcinoma sequence, generally accepted as the pathway by which most colorectal carcinomas evolve from initial adenomas.⁷⁷ Loss of APC tumour suppressor gene function is thought to be one of the first genetic changes in colorectal adenoma development. Patients with FAP have an autosomally dominant inherited germline mutation of APC on chromosome 5q21⁷⁸ and are therefore susceptible to mutation of the remaining wild-type APC allele (that is, in the “two hit” loss of tumour suppressor gene hypothesis).⁷⁹ This LOH of the wild-type APC allele can occur by either a germline mutation or somatic mutation, both causing truncating APC mutations,⁸⁰ and the development of a spontaneous microadenoma, which may further progress to a macroscopic adenoma, and subsequent colorectal carcinoma. Hundreds of specific APC mutations have been characterised, and the position of the mutation appears to dictate the severity and onset of FAP.⁴⁶ In addition to its established role in the degradation of β -catenin and regulation of proliferation of the target genes downstream of the Wnt pathway, APC is also vital in establishment of epithelial cell polarity during mitosis via direct interactions with microtubules,⁸¹ and regulates asymmetrical stem cell division in *Drosophila* to maintain a balance between stem cell self renewal and the production of daughter cells committed to differentiation.⁸² Therefore, mutation of APC and the resultant development of colorectal neoplasia may result from the misalignment of the mitotic spindle and centrosomes during intestinal stem cell division, leading to an imbalance in asymmetrical cell division and increased cell proliferation.

Top down or bottom up microadenoma formation

There are currently two proposed morphological pathways of spontaneous microadenoma development, and gastrointestinal stem cells are important players in each. In the “bottom up” theory, a stem cell situated in a niche in the crypt base acquires a second mutation in the APC gene, and expands stochastically producing neoplastic daughter cells, which migrate upwards to colonise the entire crypt and form a clonal monocryptal adenoma.¹⁹ These dysplastic crypts replicate and expand predominantly by crypt fission wherein they bifurcate and form buds in the crypt base, which ascend longitudinally to produce mutant monoclonal daughter crypts.^{83–84} Interestingly, cells with a single APC mutation in non-adenomatous mucosa in FAP show a large increase in the incidence of crypts in fission,⁸³ and crypt fission is also believed to be the means of expansion of hyperplastic polyps,⁸⁵ and of aberrant crypt foci, the proposed precursors of colorectal adenomas.^{86–87} In the second theory, the “top down” hypothesis, an initial stem cell mutation is proposed to occur in the epithelial mucosa situated between two crypt orifices, the “intracryptal zone”, with subsequent stem cell division producing a mutant clone which expands laterally and downwards into the crypt, displacing the normal epithelial cells.⁸⁸ However, as discussed, the bulk of evidence

Figure 7 (A) Contrasting theories for the morphogenesis of adenomas and the part played by stem cells. (A) Haematoxylin and eosin stained sections of a small tubular adenoma. Dysplastic epithelium is superficial within the crypts, with histologically normal underlying epithelium. (B) Abrupt transition between dysplastic and normal appearing epithelial cells at the mid point of this crypt. Proliferative activity assessed with the Ki-67 antibody distributed throughout the dysplastic epithelium at the top of the crypts. (C) Nuclear β -catenin is highly expressed and distributed throughout the dysplastic epithelium at the top of the crypts but not in the crypt bases. (D) β -Catenin in the nuclei of adenomatous crypts from a tiny tubular adenoma. (E) Nuclear β -catenin extends to the bottom of crypts in early adenomas, including the very bases of the crypts. (F) β -catenin staining in nuclei of budding crypts (A–F, from Shih and colleagues⁸⁸ with permission). (G) Junction between early adenomatous crypts, showing a sharp junction on the surface with accumulation of nuclear β -catenin, giving way sharply to membranous staining in the normal surface cells. (H) High power serial sections, demonstrating the sharp junction between nuclear staining in the adenomatous cells and membranous staining in normal surface epithelial cells. (I) Surface continuity between crypts showing nuclear β -catenin staining. (J) Crypts from a larger adenoma stained for β -catenin showing invasion of adjacent crypt territories in a top down fashion (G–J, from Preston and colleagues⁹¹ with permission).

indicates that the gastrointestinal stem cell(s) are located in the base of the crypt (reviewed in Wright⁷), with no indication of a stem cell population in the intracryptal zone, and so a modified top down hypothesis is proposed that a stem cell in the crypt base acquires a mutation and subsequently migrates to the intracryptal zone whereupon it undergoes neoplastic expansion.⁸⁸ It is possible that both (“top down” and “bottom up”) pathways occur as there is evidence for both mechanisms of monoclonal adenoma morphogenesis.⁸⁹

The top down hypothesis is based on investigations of early non-FAP adenomas where dysplastic cells were observed exclusively at the orifices and luminal surface of colonic crypts. Half the sample showed LOH for *APC* in the upper portion of the crypts and additionally, only superficial cells showed significant proliferative activity (fig 7C). The observed nuclear localisation of β -catenin verified the loss of function of a gene in the *Wnt* pathway, most likely *APC*,

and indeed an *APC* mutation was only present in these apical cells,⁸⁸ consistent with previous morphological studies.⁹⁰ However, in a study supporting the bottom up hypothesis, small (<3 mm) tubular colorectal adenomas also displayed nuclear accumulation of β -catenin (fig 7G) although the observed nuclear β -catenin expression was present in cells extending to the base of the crypts (fig 7E, F) and in crypts undergoing fission, with marked expression in the nuclei of buds in the crypt base (fig 7H). Towards the luminal surface of the crypts, there was a distinct cut off between the adenomatous cells showing nuclear β -catenin and those surface cells which did not (fig 7E, F). Adjacent crypts were filled with dysplastic cells expressing nuclear β -catenin, which were not confined to the upper portions of the crypts. However, in larger adenomas, there was unequivocal evidence of cells growing down from the intracryptal zone, between two crypt orifices, replacing epithelial cells within normal non-dysplastic crypts (fig 7J). In these large

Figure 8 Microdissected crypts from normal colonic mucosae and adenomas. (A) Symmetrical fission of normal colonic crypts. (B) Isolated adenomatous crypt showing frequent crypt fission with atypical and asymmetrical branching. (C) Another crypt from an adenoma with a bizarre shape, asymmetrical branching, and multiple budding (A–C, from Preston and colleagues⁹¹ with permission).

adenomas, multiple asymmetrical fission events were frequently observed with budding from the superficial and mid crypt (fig 8B, C) whereas crypt fission was rare in normal and non-involved mucosa, usually beginning with a basal bifurcation (fig 8A).⁸⁵

In a rare XO/XY patient with FAP, mentioned previously, small monocryptal adenomas (<2.5 mm) were monoclonal, displaying either an XO or XY genotype (fig 9A). However, 76% of adenomas greater than one crypt in size displayed a mixed karyotype of XO and XY cells and were therefore polyclonal,¹⁹ with a sharp boundary at the luminal surface between adjacent adenomatous crypt territories (fig 9B).⁹¹ According to the bottom up mechanism, expansion of a clonal monocryptal adenoma would lead to the formation of a monoclonal microadenoma, and adenoma, although the observed polyclonal adenomas in this patient could be a result of adenomatous growth in non-involved neighbouring

crypts induced by transformed stem cells in early adenomas, or they may indeed be true polyclonal proliferations caused by the clustering of transformed stem cells.¹⁹ The latter is in accordance with the “field cancerisation” hypothesis wherein an initial carcinogenic stimulus is thought to induce neoplastic transformation in multiple cells within a tissue which proliferate to produce a polyclonal neoplastic lesion.⁹²

Studies of older adenomas have shown that cells towards the top of the crypt display maximum proliferative activity,^{93–94} with increased apoptosis in cells of the crypt base,⁹⁵ indicating that migration kinetics are reversed and cells migrate towards the crypt base, possibly from a stem cell in the intracryptal region (that is, the top down hypothesis). However, analyses of the methylation histories of colorectal adenomas have suggested a stem cell architecture indicative of a bottom up mechanism and moreover, mitotic events are evenly distributed throughout the cells of adenomatous crypts and therefore do not suggest concentration of dysplastic cells in the tops of the crypts.⁹⁶ Computer modelling studies suggest that an expanded stem cell population within a crypt in FAP causes an upward shift in the proliferative compartment towards the top of the crypt,^{97–98} and it has previously been suggested that an expanded stem cell population may produce an increase in the rate of crypt fission.⁹⁹ Crypt fission is therefore important in the expansion of mutated clones in adenomas. While the morphology of this process is quite distinct, the molecular mechanisms that govern it are far from clear. We conclude that the initial event in the genesis of colorectal adenomas is the monocryptal adenoma, where initial growth occurs via crypt fission, and spread into adjacent crypt territories is a later secondary event.

CONCLUSIONS

The gastrointestinal tract is a highly specialised tissue which undergoes constant cell turnover relative to the demand imposed upon it by damage or disease. Although unidentified, it is generally accepted that epithelial gastrointestinal stem cells are situated within a niche in the base of the crypt or gland, produced and maintained by the subjacent mesenchymal cells, which regulate stem cell function by paracrine secretion of regulatory growth factors and cytokines. The gastrointestinal stem cell produces all the adult cell lineages of the gastrointestinal mucosa, and is thereby perceived as the most important regulatory element in gastrointestinal function. Despite this status, gastrointestinal stem cells are unidentified and their quantity and location within each gastric gland and intestinal crypt is inconclusive. A large body of evidence suggests that under normal circumstances in both mice and humans, gastric glands and intestinal crypts are monoclonal structures produced by a single multipotential stem cell with the ability to transdifferentiate and form all the specialised lineages which comprise the gland or crypt. However, in gastrointestinal neoplasia, although most monocryptal adenomas appear clonal in their derivation, larger adenomas and carcinomas appear to be chiefly polyclonal, possibly arising from a proliferation of multiple mutated stem cells, or by the convergence of multiple monoclonal microadenomas. The morphological pathways of gastrointestinal neoplasia are also debated, with two main conflicting hypotheses of the expansion of a mutated clone, the “bottom up” and “top-down” theories, although both concur that gastrointestinal neoplasia is initiated by the formation of a monocryptal adenoma which

Figure 9 Adenomas from an XO/XY mosaic individual with familial adenomatous polyposis, stained by in situ hybridisation for a Y chromosome specific probe (from Preston and colleagues⁹¹ with permission). (A) Clonal monocryptal adenoma. (B) Polyclonal adenoma, with a mixture of XO and XY crypts. Note the sharp margin between the territories of XO and XY adenomatous crypts at the surface, with no evidence of invasion.

divides and spreads by crypt fission. The molecular pathways governing stem cell proliferation and cell fate and patterning in the gastrointestinal mucosa are emerging, and provide an additional insight into the genetic events in gastrointestinal neoplasia, as mutation of the genes in these pathways are frequently present in dysplastic gastrointestinal cells. With the observation that transplanted bone marrow stem cells can transdifferentiate to form adult lineages in the mouse and human gastrointestinal tract, a new concept of stem cell biology in the treatment of human disease has emerged. Transplanted BMSC make a large contribution to the myofibroblasts, fibroblasts, and smooth muscle cells in the intestinal lamina propria and mucosal layers, with a significant increase in colitis. This propensity for regeneration of damaged tissue presents the adult BMSC as a therapeutic tool in the treatment of human disease, such as inflammatory bowel disease. It is important to decipher the mechanisms of this observed adult stem cell plasticity—that is, the ongoing debate of “transdifferentiation versus spontaneous fusion”—and the propensity for these bone marrow derived cells to clonally expand and possibly contribute to gastrointestinal stem cell populations.

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