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Stem Cell Review

The intestinal epithelial stem cell: the mucosal governor

CHRISTOPHER S. POTTEN, CATHERINE BOOTH AND D. MARK PRITCHARD*†

*CRC Department of Epithelial Biology, Paterson Institute For Cancer Research, Christie Hospital NHS Trust, Withington, Manchester, *CRC Molecular and Cellular Pharmacology Group, School of Biological Sciences, University of Manchester, Manchester and †Department of Medicine, Hope Hospital, University of Manchester, Salford, UK*

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Summary. All epithelial cells in the small and large intestine are thought to originate from stem cells located towards the base of the crypts of Lieberkühn. To-date, there are no specific intestinal stem cell markers, hence stem cell properties can only be inferred. A range of experimental techniques have been employed including cell position mapping, radiation regeneration (clonogenic) assays, chimeric and transgenic mice. This review discusses the implications of experiments performed using these techniques in order to deduce the number, location and functional properties of stem cells. Stem cell homeostasis is maintained by cell proliferation and death 'through apoptosis'. The various growth and matrix factors and genes which may control these processes, and be important for stem cell function, are discussed along with their carcinogenic and clinical implications.

Keywords: stem cell, intestine, apoptosis, epithelium

Proliferative organization of the intestinal epithelium

The lumenal surface of the intestine is lined by a simple columnar epithelium. The surface of both the small and large intestine is folded into a number of deep cavities or crypts of Lieberkühn, which are embedded in connective tissue. In the small intestine the surface area is further increased by finger-like projections, or villi, also covered by epithelium, which protrude into the gut lumen. Although the crypts are approximately 10× smaller than the villi, they are much more numerous (7× more in the duodenum, 4× more in the ileum, Wright & Alison 1984). The effect of these numerous 'pits and projections' is to form a huge surface area. This surface area is further expanded by the presence of a carpet-like covering of microvilli on the apical (lumenal) surface of the columnar epithelial cells.

The functional cells of the epithelium are located predominantly towards the lumenal pole of the crypt-villus axis (i.e. on the villi of the small intestine and at the top of the crypts of the large intestine). Once they have fulfilled their differentiated function (brush border enzyme secretion, mucus secretion, nutrient and water absorption) the cells senesce and are sloughed off into the lumen itself, possibly after initiating some aspects of the

Correspondence: C.S. Potten, CRC Department of Epithelial Biology, Paterson Institute For Cancer Research, Christie Hospital NHS Trust, Withington, Manchester, M20 4BX, UK. Fax: +44 (0) 161 4463181, E-mail: epbcsp@picr.man.ac.uk

apoptosis programme. There is therefore a continuous loss of cells which, in order to maintain epithelial integrity and sustain a protective barrier, must be replaced at an equal rate. This epithelium is one of the most rapidly renewing tissues in the body and has a very large number of cycling cells. These new cells are produced in the bottom two-thirds of the crypts where up to 60% of the total cells of the crypt can be passing through the cell cycle twice a day, i.e. approximately 150 cells of the total 250 cells of a mouse small intestinal crypt. The zone of these cells in the crypt is therefore frequently referred to as the proliferative zone.

In a healthy adult animal, the rates of cell production and loss balance exactly and must be under extremely stringent controls. In situations of decreased proliferation (or increased cell death) the epithelial integrity will be lost, whereas increased proliferation (or reduced levels of cell death) will increase the cell number. The success of the controls normally regulating this process can be illustrated by the fact that although about 10^{10} cells are replaced daily in an adult human small intestine, cancers of this tissue are relatively rare (Potten 1995a).

The effect of the spatial separation of the proliferative and differentiated (functional) units is to produce a continuous linear migration of 'new' cells from the lower crypt upwards to the lumenal surface (except for the Paneth cells of the small intestine, which result from migration downwards to the crypt base). The topography of the tissue can therefore be a useful indicator of cellular age, with the youngest cells (and therefore the stem cells) located towards the crypt base. It is possible to monitor the frequency of particular events (DNA synthesis, mitosis, apoptosis) or cell types at the different positions along this polarised axis, and relate the ability of the cell at each position, to undertake a particular event or have that particular phenotype (Figure 1).

Such studies have clearly shown that cells in the upper region of the crypt undergo only one further mitotic division after incorporating label during S phase, i.e these are cells with a very limited division potential. Cells slightly further down the crypt, which migrate later on to the villus, dilute their thymidine labelled DNA in a manner consistent with two rounds of division. Similarly, cells yet further down the crypt have diluted their label consistent with three rounds of division. Such label dilution studies cannot practically be taken any further, but these observations do show that cells in the upper region of the crypt have a limited and defined number of divisions. The mechanisms determining this limit remain uncertain (deterministic or stochastic; inherent cell counting or cell position determination).

Such tracking experiments also show that the complete

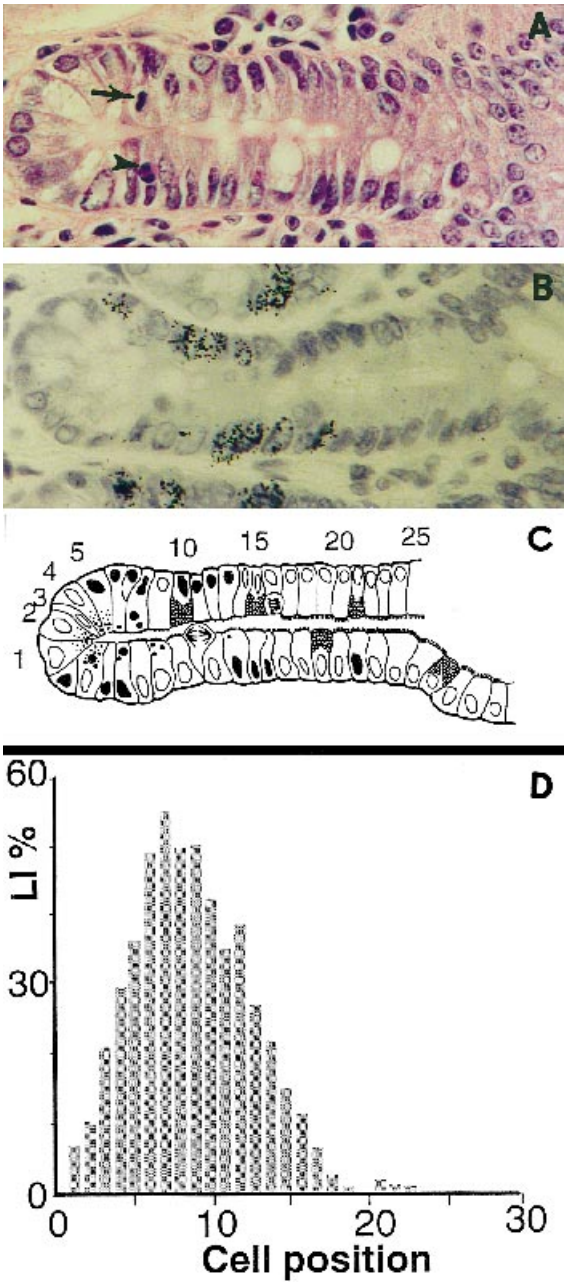

Figure 1. Evaluation of positional frequency of S phase and apoptotic cells. The photographs show longitudinal sections through small intestinal crypts. A, the apoptotic cell is indicated by the arrowhead, the mitotic cell indicated by the arrow. B, after autoradiography black silver grains overlay the nuclei that incorporated tritiated thymidine during S phase. C, the cartoon D, illustrates how the position of such events up the crypt axis can be determined (position 1 being the crypt base). When a number of crypt cross sections are counted the event frequency at each cell position can be plotted (e.g. thymidine labelling).

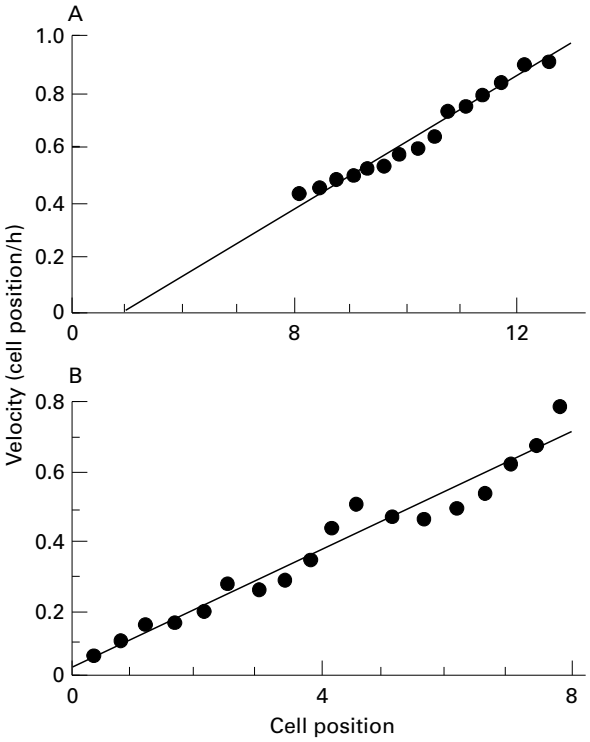

Figure 2. Changes in cell velocity in the crypt as analysed by comparing successive percentiles in 40 min and 3 h post tritiated thymidine labelled samples of unirradiated adult mice. The point of origin of cell migration in small and large intestine can be extrapolated from these curves. A, Small intestine (4.9 ± 0.2); B, Large intestine (-0.2 ± 0.2) Redrawn from Qiu *et al.* (1994).

life expectancy of a cell born from division in the upper region of the crypt is about two to three days, after which it is shed into the lumen. Studies using early time intervals after labelling S phase cells have been used to measure the migration velocity of cells in the crypt and the variation in this velocity with cell position (Kaur & Potten 1986). These experiments showed that in the small intestine the extrapolated origin of this migration was found at about cell position 4 (4 cells up from the crypt base). A more detailed and extensive study looking at the changing position of various percentiles in the labelling index confirmed that the extrapolated origin was to be found a few positions from the base of the crypt in the small intestine, and essentially at the base of the crypt in the mid-colon (Qiu *et al.* 1994), Figure 2. The origin of these cell migration vs. cell position plots represents the point in the crypt from which everything else is ultimately derived, i.e. the position of the stem cells.

Differentiated phenotypes

As a cell moves through the proliferative zone it becomes increasingly differentiated. In the small intestine, an immature proliferating cell, often referred to as a transit cell, has the option to become one of five different mature phenotypes, namely Paneth cells, goblet cells, endocrine cells, M cells and absorptive enterocytes. The Paneth cells are normally only located in the small intestine, hence the colon contains only four lineages. Similarly, the M cells are only located close to the Peyer's patch lymphoctes, and hence this lineage is absent in crypts that are distant from these foci.

Little is known about the function of Paneth cells which are located at the crypt base, although they are thought to perform an antibacterial function. Their extensive endoplasmic reticulum and secretory granules allows them to secrete large quantities of proteins such as lysozyme, TNF-α and cryptidins (a kind of defensin) into the crypt lumen – which may contribute to the low bacterial content in this part of the gastrointestinal tract, although the antibacterial properties of these cells have not been proven (Deckx *et al.* 1967; Ouellette *et al.* 1989; Tan *et al.* 1993; Harwig & Eisenhauer 1995). Paneth cells also contain phospholipase-A2 (enhancing factor) which may be involved in regulating apoptosis (Mulherkar *et al.* 1993). The close proximity of Paneth cells to the stem cells has led some to speculate that Paneth cells may have a role in nurturing, regulating or protecting stem cells, but this again is currently conjecture. However, certain species such as dog and pig appear to lack these cells altogether, suggesting that they do not perform such an essential function (see Figure 3). The actual location of the stem cells in the small intestinal crypt is thought to be the consequence of the presence or absence of Paneth cells – the stem cells being higher due to the Paneth cells at the crypt base. The exact point during cell maturation at which the Paneth cells appear is also unknown, as is whether their progenitors move immediately downwards (Hampton 1968), or initially move upwards with the general cell flow and then move down to the crypt base. However, the fully differentiated Paneth cells appear after 3 days (Cairnie 1970) and, unlike the other upwardly moving cell types, have a much longer lifetime within the animal, lasting for over two weeks (when they appear to be removed by phagocytosis, Troughton & Trier 1969; Cheng & Leblond 1974a). The intercalated cells (non-Paneth cells located near the crypt base) may be early Paneth cells (Hampton 1968), although some have argued these are actually stem cells (Bjerknes & Cheng 1981). If this were

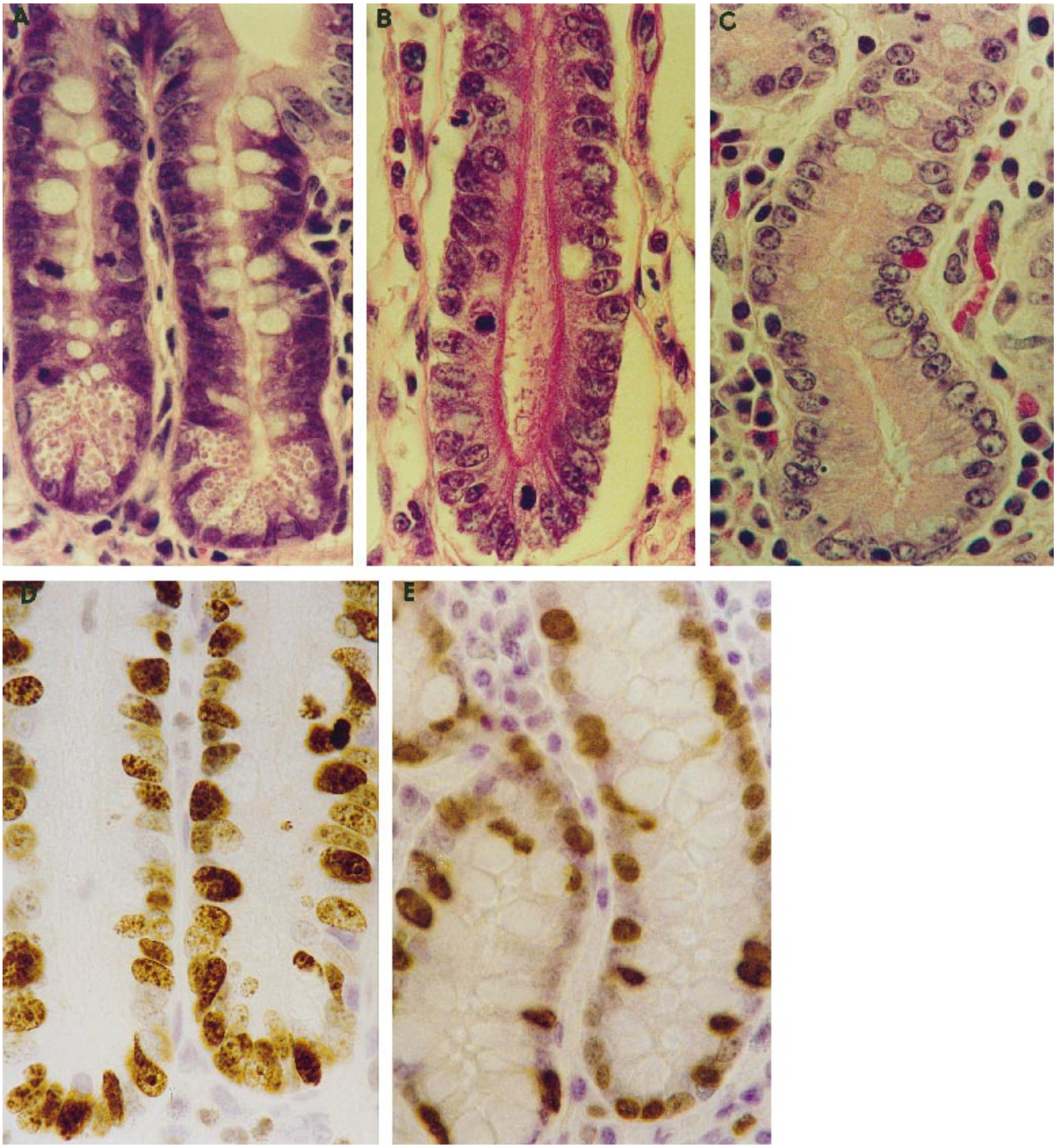

Figure 3. Paneth cells are not present in all mammalian species. Although present at the base of the small intestinal crypt in mouse (A) and man (not shown), this cell type is not present in the small intestine of dogs (B) or pigs (C). In the absence of Paneth cells the proliferative cells are located at the crypt base. Ki67 labelling reveals the presence of proliferative cells at the very base of the small intestinal crypts in both dog (D) and pig (E). (Mag $\times$1000).

true, the cells would have to divide and therefore would occasionally be observed in pairs. They would also need to generate very complex cell migration pathways in this critical zone of the crypt. As it is very rare to see pairs of intercalated cells, this possibly points more towards these cells being pre-Paneth cells (Chwalinski & Potten 1989).

Also poorly defined are the M or microfold cell lineage

(reviewed by Neutra *et al.* 1996; Savidge 1996). These constitute some of the epithelial cells that overlay the lymphoid tissue (Peyer's patches) within the gut. The M cells forming part of the epithelium in this region are a barrier that is reasonably easy for the lymphocytes to breach and for antigen sampling to occur. Lymphocytes can frequently be observed within the folds or 'pockets' of the M cell membranes – the invaginations effectively reducing the distance the lymphocyte has to travel. The signals for this specific morphology and behaviour are therefore presumably the cytokines derived from the mass of lymphocytes they overlay.

Goblet cells are flask shaped cells that do not have transporting apical microvilli but produce the mucins that protect the epithelial surface and ease the passage of food along the gut. They first appear in the proliferative zone of the crypts, after approximately 3 divisions, although in the small intestine it appears that 5 or 6 divisions are needed before the fully differentiated cell is produced (Paulus *et al.* 1993) (i.e these cells appear about 2 divisions from the end of the lineage). This linear differentiation pathway may differ from that of the endocrine cells (also called enteroendocrine cells) which contain neurosecretory granules and release peptide hormones.

Endocrine cells are much more sparse than goblet cells, comprising only 5–10 cells per crypt. These cells appear to branch into at least two subsets of cells containing different sets of neuropeptides (Roth *et al.* 1992). The presence of the neuropeptides originally led to the belief that these cells were actually derived from the neural crest, but this was disproved by sex chimera experiments (Thompson *et al.* 1990). Others have suggested that Goblet and endocrine cells share a common ancestor – the so-called amphicrine cells which share characteristics of both cell types (Cheng & Leblond 1974b).

By far the majority of cells are the absorptive enterocytes of the small intestine, or colonocytes of the colon. The microvillus brush border develops as the cells differentiate in the upper crypt and becomes larger and more numerous in the more mature cells. This apical surface of the cell therefore comprises a huge area across which molecules and ions can be rapidly transported.

Finally, a novel sixth lineage has been described in tissue in which the mucosal barrier has been breached – an ulcer associated lineage (Wright *et al.* 1990). Not normally generated, this phenotype appears to have a specialized antigenic profile and performs a specific function. The lineage arises after mucosal damage and appears to form as a branching duct from an existing crypt close to the wound site. These cells are therefore derived from existing crypt stem cells, and appear to secrete mucins, epidermal growth factor and trefoil peptides.

Within the stomach, four differentiated phenotypes are derived from pluripotent stem cells – chief (digestive enzyme secreting), endocrine (hormone secreting), parietal (acid secreting) and mucus secreting cells (Wright & Alison 1984; Canfield *et al.* 1996). In the proximal stomach (fundic mucosa) the 'crypts' are long tubular structures that can nominally be divided into three regions: the base or fundus, the mid or neck region, and the upper isthmus region. Generally, the chief cells reside towards the base and the parietal and mucous cells towards the lumen. In the distal pyloric region the glands appear to contain more mucous cells but fewer parietal cells and no chief cells (Wright & Alison 1984). The origin of these cells appears to be a stem cell compartment located in the mid-crypt region, which initially give rise to proliferating, immature phenotypes. However, little is known about stem cell numbers and behaviour in this region of the gastrointestinal tract, and therefore this review will concentrate on the stem cells of the intestinal epithelium.

Problems estimating stem cell number

It is now generally accepted that the differentiated epithelial cell types are derived from progenitor cells (stem cells) located at, or just above, the very bottom of the crypt (Potten & Loeffler 1990). Unfortunately, there are, as yet, no stem cell markers and so most of what is currently understood has been extrapolated from experiments in which past or future events are monitored following crypt perturbations. The origin of such crypt responses always appears to be towards the base of the crypt in the small intestine (S.I.) (around 4–6 cell positions up from the base), and at the very base (positions 1 and 2) in the large intestine (L.I.). For example, following the cytotoxic damage and reproductive sterility induced by large doses of radiation there is clear evidence that the proliferative regenerative response is initiated in cells located at the position attributed to stem cells (Potten *et al.* 1990; Al-Dewachi *et al.* 1977, 1980). Similarly, when the bases of the crypts were irradiated with appropriately filtered weak beta particles from ^{147}promethium the crypts could be sterilized by a dose of irradiation that spares the mid and upper crypt cells from exposure to the sterilizing dose (Hendry *et al.* 1989). Thus, the upper crypt cells do not have the capacity to regenerate a crypt following radiation injury, i.e. the regenerative clonogenic cells must be located in the lower regions of the crypt. The undifferentiated cells found at positions 4–6 (S.I.) or 1–2 (L.I.) are also the origin of the cellular migration

pathway in the small intestine (Kaur & Potten 1986; Qiu *et al.* 1994). It is likely that the origin of this migration is mid-crypt (mid-gastric gland) in the stomach and there is therefore a bi-directional flow of cells in this region of the GI tract. Some have also proposed a similar situation exists in the proximal colon (Kovacs & Potten 1973; Sato & Ahnen 1992).

The actual number of stem cells in an intestinal crypt has been, and still is, a matter of considerable debate. This cell number has far reaching implications on a number of crypt parameters: the cell cycle time of a functional stem cell; the number of divisions a cell must undergo before it becomes fully differentiated; the number of lineages an individual stem cell normally generates or is capable of generating; the number of cells capable of tissue regeneration following damage and the number of potential cancer forming cells.

Some have consistently maintained that the majority of proliferating cells are stem cells, which would suggest that 60% of the cells in a crypt might be stem cells. At the opposite extreme others have claimed that the crypts are monoclonal i.e contain a single stem cell (0.4% of crypt cells) from which all the other cells are derived (Ponder *et al.* 1985; Schmidt *et al.* 1988; Winton *et al.* 1988; Roth *et al.* 1991). Many of these estimates have been based upon the aforementioned interpretations of the responses of damaged/disturbed crypts – treatment which itself may alter normal stem cell properties to differing degrees, and result in differing estimates of cell number (Potten & Loeffler 1990). This has been likened to Heisenberg's uncertainty principle in quantum physics – which states, in simplified terms, that the actual act of measuring a parameter results in its alteration, and therefore introduces an uncertainty into the measurement (Potten & Loeffler 1990). An additional problem when considering stem cells is the term itself – many different interpretations have been used in the literature. As stem cells cannot be defined by their morphology or by the expression of certain genes they must be defined by their function. It was proposed in 1990 by Potten and Loeffler that stemness was not necessarily a single property, but a number of properties or options which such a cell has the capability of executing depending on the circumstances. It is now generally accepted that these properties are; that the cell must be an undifferentiated cell type that is capable of proliferation, self-maintenance, producing many differentiated progeny and regenerating the tissue after injury. It must also retain the ability to switch between these options when appropriate.

The term undifferentiated is probably the loosest term in this definition, being a relative term. In the case of the crypt it is simply relative to the other epithelial cells in this tissue – although these undifferentiated stem cells are probably differentiated relative to embryonic stem cells (i.e. not capable of producing an embryo, or even other tissues).

Self-maintenance refers to the ability of the stem cells to maintain their own population numbers. Thus, when a stem cell undergoes mitosis it must be able to produce at least one daughter that remains as a stem cell. This may be via an *asymmetric division* where one daughter may go on to divide, mature and differentiate. Should both daughter cells become stem cells – *symmetrical division* – the stem cell population would inevitably double, whereas if both became maturing cells the stem cells would disappear. In an ever changing environment it is likely that stem cells have the ability to switch between each of these options in order to adapt to proliferative or death induced changes within the crypt, with the asymmetrical division being the 'average' response under steady state conditions [i.e. the probability of one daughter becoming a stem cell (the probability of self maintenance) is 0.5]. By switching between the options the crypt can regulate stem cell number and hence crypt size. The molecular signals that trigger this switching remain to be identified.

Using such criteria, crypts may have a number of true or *actual stem cells*, functioning as such in the steady state situation. However, there may be many more cells – *potential stem cells* – capable of stem cell function if necessary (i.e. following damage). These are most likely to be the first descendants of the actual stem cells. Thus, there may be a limited number of operational or actual stem cells, but many more clonogenic cells (all the cells *capable* of acting as stem cells, to produce a clonogenic unit or crypt after damage to the crypt and its stem cells) (Potten & Hendry 1985).

Polyclonal vs. monoclonal crypts

The number of symmetrical divisions that occur during the development of the adult crypt from a hypothetical single cryptogenic stem cell has not been established. However, it has been estimated from mathematical modelling using experimental data that a normal steady state adult mouse small intestinal crypt contains four to six actual stem cells, implying that more than two symmetrical divisions occurred during the development of the crypt (Potten & Loeffler 1990). These cells appear to be located around four or five positions up from the crypt base, scattered amongst their daughters in the circumferential ring of about 16 cells (small intestine).

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Figure 4. The cellular output from a single crypt can be detected in heterozygous mice which incur a mutation at the *Dolichos biflorus* gene locus. The brown stain (peroxidase-DAB) is due to the binding of this lectin. Mutations in the stem cells result in crypts and villi containing negative linear stripes – the cells are no longer able to bind the lectin (A). The stripes can show sudden changes in width (B), indicative of a varied number of negative cells moving from crypts on to the villus. This is consistent with changes in the number of mutated stem cells in the crypts as suggested by the competitive multistem cell hypothesis. We are indebted to Y.Q.Li for these photographs.

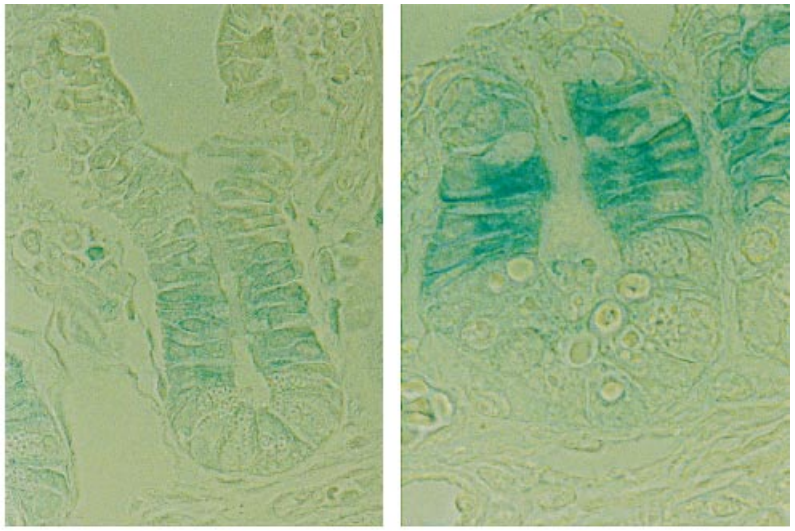

Figure 5. β-galactosidase (lacZ) expression in the small intestine of ROSA 11 mice. The blue staining is confined to the proliferative cells (there is no villus or Paneth cell labelling). It can be seen that the staining is not restricted to the stem cell region (positions 4–6). We are grateful to A.J.Merritt and W. Dove for help in producing these photographs.

Other estimates of stem cell number have been more conservative, and some have argued for the existence of a single lone stem cell. For example, chimeric mice in which only one strain expresses a *Dolichos bifluros* lectin binding ability have been used to show that as the crypts develop over the first 14 postnatal days they become exclusively positive or negative for the lectin binding. There are no mixed crypts – indicating that each crypt *at this stage* is *derived* from a single stem cell (Ponder *et al*. 1985; Schmidt *et al*. 1988). This has been interpreted as indicating that *adult* crypts have one ultimate stem cell, a situation which may not be true. Adult mice which are F1 hybrids for *Dolichos bifloros* lectin binding, i.e. are heterozygotes and incur a mutation in this allele in certain stem cells (which then fail to bind the lectin) gradually exhibit this mutation throughout the entire crypt and go on to produce a stripe of negative staining in the villus as the crypt progeny migrate upwards, Figure 4 (Winton *et al*. 1988; Li *et al*. 1994). This does not necessarily indicate that the crypts contain a single stem cell. The same observation would have been seen if a single mutated stem cell amongst several occasionally divided symmetrically and in this way the mutated cells gradually replaced the other stem cells (which might either die or divide symmetrically to produce more differentiated daughter cells) (Loeffler *et al*. 1993). Equally, the mutated cell could be lost. This stem cell competition hypothesis would explain the long time interval between the initial mutational event and the appearance of a totally mutated (monophenotypic) crypt – an interval much longer than the cell turnover time of the crypt. Furthermore, in this situation, stripes of mutated cells with variations in width on the villus have been found – supporting a multistem cell crypt, Figure 4 (Li *et al.* unpublished observation). Additionally, it has been calculated that 10 days post mutation the stripe or 'ribbon' of mutated cells migrating up the villus in the *Dolichos bifluros* mutated mice is only two cells wide, equating to only 25% of the cellular output of a single crypt, i.e. indicative of four functional stem cells per crypt (Winton 1997). However, in these experiments mutations were often induced by agents which kill crypt stem cells (Li *et al*. 1994). This obviously disturbs the normal steady state of the crypt, and could confuse estimates of stem cell number.

Similarly mixed crypts have also been observed following mutational events in mice which are heterozygous for glucose-6-phosphate-dehydrogenase (G6PD) deficiency, and in humans carrying an O-acetyl-transferase polymorphism (Williams *et al*. 1992; Park *et al*. 1995; Wright 1996). Furthermore, the crypts containing a mixture of mutated and nonmutated cells gradually disappear, coincident with the appearance of crypts with a completely mutated phenotype (Williams *et al*. 1992). Finally, on a more physical basis, it is hard to envisage how a lone stem cell would maintain a stable, symmetrical and ordered crypt (with maintained symmetry in the Paneth cell region). Modelling exercises have shown that a single slowly cycling stem cell would cause large oscillations in crypt size and extend the proliferative zone higher up the crypt (Potten & Loeffler 1990), and this is not observed.

Further insights into stem cell behaviour from conventional transgenic mice

Gordon and coworkers have produced a number of conventional transgenic mice by pronuclear injection, using promoters from the 5′-nontranscribed domains of fatty acid binding protein genes linked to a reporter such as human growth hormone (hGH). (reviewed in Gordon *et al*. 1992; Hermiston & Gordon 1993). The mouse has three fatty acid binding protein genes, each of which has a distinctive pattern of spatial and temporal expression. *Fabpl* (liver fatty acid binding protein gene) is expressed from day 14–15 in embryonic small intestinal enterocytes, but not in the colon; *Fabpi* (intestinal fatty acid binding protein gene) is expressed from the same stage of development, but in villus enterocytes and in the upper crypts of the proximal colon; *Ilbp* (ileal lipid binding protein) is expressed in differentiating enterocytes of the ileum and caecum from postnatal days 14–15 (suckling/weaning transition).

Of particular interest have been mice generated using nucleotides -4000 to $+21$ or nucleotides -596 to $+21$ of *Fabpl* (Sweetser *et al*. 1988a,, 1988b; Hauft *et al*. 1989). The transgene is expressed in the appropriate *Fabpl* fashion, but is also inappropriately expressed in the proliferating and nonproliferating cells of small intestinal and colonic crypts as well as in all four epithelial cell lineages. This inappropriate expression has provided opportunities for

- describing the differentiation programs of certain gut epithelial cell lineages;
- identifying subtle differences in the regulatory environments of intestinal cellular populations that are not evident from an analysis of the patterns of expression of their endogenous gene products;
- demonstrating that gene expression is co-ordinated between adjacent crypts;
- illustrating that the gut epithelium has a temporal as well as spatial axis (Hermiston & Gordon 1993).

Further analysis of these transgenic mice has led to insights into both stem cell numbers and the crypt life cycle within the colon during the first year after birth

(Cohn *et al*. 1991). It was found that the number of crypts expressing the transgene decreased with increasing age, and that the silencing of transgene expression occurred in multicrypt patches. The observation that such colonic crypts were either reporter positive or reporter negative prompted the authors to suggest that in this region of the intestine the crypts were monoclonal, i.e. with lone stem cells that were either reporter negative or positive. However, the alternative stem cell competition explanation discussed above may again apply. This co-ordinated regulation of transgene expression within the patches could also reflect a deterministic event, whereby colonic crypts are resupplied with stem cells (from outside the crypts) over a period of months by a common progenitor. There is however, no evidence supporting the idea that external cells can enter a crypt. An alternative explanation is that the patch could represent the progeny of a master crypt which underwent crypt fission early in development and the stem cell properties were programmed into this master crypt's stem cell prior to crypt fission.

This wave of silencing of transgene expression does not occur in the small intestine, although one pedigree of the above transgenics displays an unusual heritable mosaic pattern of reporter expression (Roth *et al*. 1991). Wholly reporter-positive or wholly reporter-negative monophenotypic crypts gave rise to vertical coherent bands of wholly reporter-positive or wholly reporter-negative cells that produced striped villi, reminiscent of those in *Dolichos bifluros* (*Dlb-1*^{a}*/Dlb-1*^{b}*)* aggregation chimeras treated with the mutagen ethylnitrosourea described above (though the number of hGH negative crypts and stripes is many orders of magnitude greater). Statistical analysis suggested that the number of stripes per villus was fewer than the number of anatomically definable crypts surrounding the villus, indicating that transgene expression must be co-ordinately regulated between clusters of crypts. These mice have been further analysed for the evolution of villus striping during intestinal morphogenesis (Hermiston & Gordon 1995a). Striping occurred in a proximal to distal wave between birth and postnatal day 7, with no heterogeneity in the levels of hGH between cells in a coherent band, suggesting that this striping is programmed at the level of the gut stem cell.

Further work on these transgenic mice has involved the use of foetal isografts (Dubin *et al*. 1992). Isografts from day 15–16 embryos of *Fabpl*/hGH transgenic mice were implanted subcutaneously into nude mice and harvested 4–6 weeks later. These showed appropriate position specific differences in the differentiation programs of all four principal cell lineages along the cephalocaudal and crypt-villus axes, suggesting that the multipotent stem cell was able to retain a positional address in the absence of lumenal signals. Additional support for this positional address comes from analysis of *Min* mice (Moser *et al*. 1992) – adenomas contained small foci of differentiated cells representing each of the gut's four lineages and immunohistochemical analysis of their patterns of fatty acid binding protein expression correlated with the site of tumour origin along the cephalocaudal axis.

The same group have also used *Fabp* transcriptional regulatory elements to create transgenic mice expressing SV40 T antigen, human K-ras and a dominant negative mutant of human *p53* in postmitotic enterocytes (Kim *et al*. 1993). These mice had no detectable phenotype and only caused a modest increase in tumour number when crossed with *Min* mice. The lack of effect represents the remarkable protective effect of a continuously and rapidly renewing epithelium and suggests that models of neoplasia would require expression of gene products in crypt stem cells rather than in villous enterocytes.

Wong *et al*. (1996) transfected 129/Sv ES cells with cDNA for human APC under a *Fabpl* promoter and introduced this into C57BL/6-ROSA 26 blastocysts. This caused markedly disordered cell migration but had no effect upon proliferation or apoptosis. ROSA 26 mice were generated from promoter trap experiments in ES cells (Friedrich & Soriano 1991). Adult mice express lacZ throughout the crypt-villus and gastric-colonic axes. This allows a dual marker system to be employed (in addition to lectin UEA-1), and has advantages over lectin staining since the latter is absent in both small intestinal and colonic crypts and also in embryonic intestine. Another strain of ROSA mice (ROSA 11) has also been used successfully (Gould & Dove 1996) of the crypts of the small and large intestine. We have recently demonstrated that lacZ expression is not confined solely to the stem cell zone in ROSA 11 mice, but also extends into the early proliferative compartments (Booth *et al.* unpublished observation, Figure 5). Its promoter is therefore unlikely to represent a specific stem cell marker.

These transgenic experiments have therefore provided additional data to confirm that intestinal crypts may be monoclonal in certain circumstances while villi are polyclonal. They have also suggested that there are both temporal and spatial (duodeno-colonic and crypt-villus) axes of gene expression in the intestine and have confirmed that gene expression in postmitotic enterocytes has little long-term phenotypic effect.

Clonogenic cell estimates from in vivo regeneration models

The ability of a stem cell to regenerate the intestinal epithelium after injury has been exploited as a clonal regeneration assay which can be used to make predictions about stem cell number and function and how this function may be manipulated by growth factors to therapeutic advantage. The usefulness of this type of stem cell functional assay was first described by Till & McCulloch in 1961 as a spleen colony assay for haemopoietic stem cells. The philosophy was then adapted to the gut as a macro (late times) and then micro (early times) colony assay (Withers & Elkind 1969, 1970; Potten & Hendry 1985). This either measures the gross morphological regeneration of the denuded gut following high dose irradiation (macro) or the regeneration of individual crypts (micro). Thus, by using doses of irradiation that will reduce crypt stem cell numbers, one can measure the ability of the crypt to survive and regenerate, and the speed with which it does so. This is interpreted as being whether or not one or more clonogenic cells survived in a crypt. With certain limited assumptions the data can be used to estimate the number of potential stem cells per crypt. Using appropriate doses of irradiation or other cytotoxic agents the crypt can be reduced to a single surviving clonogenic cell and the resultant regenerated crypt will therefore be a clone.

Using clonal regeneration studies following exposure to cytotoxic agents (radiation or drugs), a hierarchical stem cell organization has been proposed for the mouse small intestine (Potten & Hendry 1995), Figure 6. This has been complemented by positional analysis of apoptosis in the crypts following cytotoxic insults (Potten 1977; Hendry *et al.* 1989). Three apparently distinct categories of stem cells have been suggested. As mentioned above, in the steady state the small intestinal crypt appears to contain 4–6 *actual* stem cells. These cells are located at the putative stem cell position 4 cells up from the base of the crypt and appear to be very sensitive to DNA damage and are unable to repair such damage. All of these cells are killed and die by apoptosis within about 6 h of 1Gy of γ irradiation. This may serve to protect the integrity of the DNA of these cells, which is essential when the longevity of this population is considered (any mutation may be continuously passed on to daughter stem cells until the animal dies). These cells experience the ultimate form of protection from mutational damage and carcinogenic transformation – altruistic death or suicide.

Using split dose techniques (Hendry 1979) the total number of clonogenic cells can be estimated. These

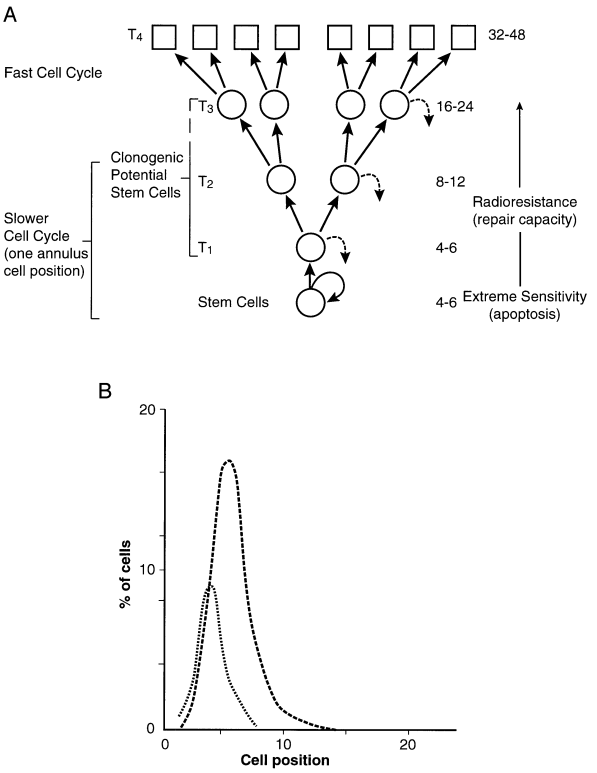

Figure 6. Crypt stem cell hierarchy. Crypts posses 4–6 actual stem cells, but many more clonogenic cells which are capable of stem cell function. A, the circles indicate clonogenic cells (including the actual stem cells). These are thought to have a three tiered radioresistance or repair capacity (see text) with the actual stem cells being very radiosensitive. B, the actual stem cells are restricted to a narrow band of cells, predominantly at positions 4–5. When the total number of clonogenic cells are considered the width of the band is greater. We are grateful to M.Loeffler and U. Paulus for this frequency plot which is based on mathematical modelling.

results indicate that after low levels of cytotoxic stress (less than 9Gy) there appear to be about 6 additional clonogenic cells per crypt (Roberts *et al.* 1995), see Figure 7. The crypt therefore possesses 6 ultimate or actual stem cells of high sensitivity and a further 6 clonogenic cells with lower sensitivity. The model proposes that the readily deleted ultimate cells are easily replaced by these 6 daughter cells – which therefore make up a second tier of stem cells. Such cells would normally become dividing transit cells and ultimately differentiate. However, at this early stage of their life they retain stem cell properties but also have the ability to repair their DNA. Hence, in a situation where the actual stem cells are killed, these cells may regenerate this first tier compartment and eventually the crypt itself.

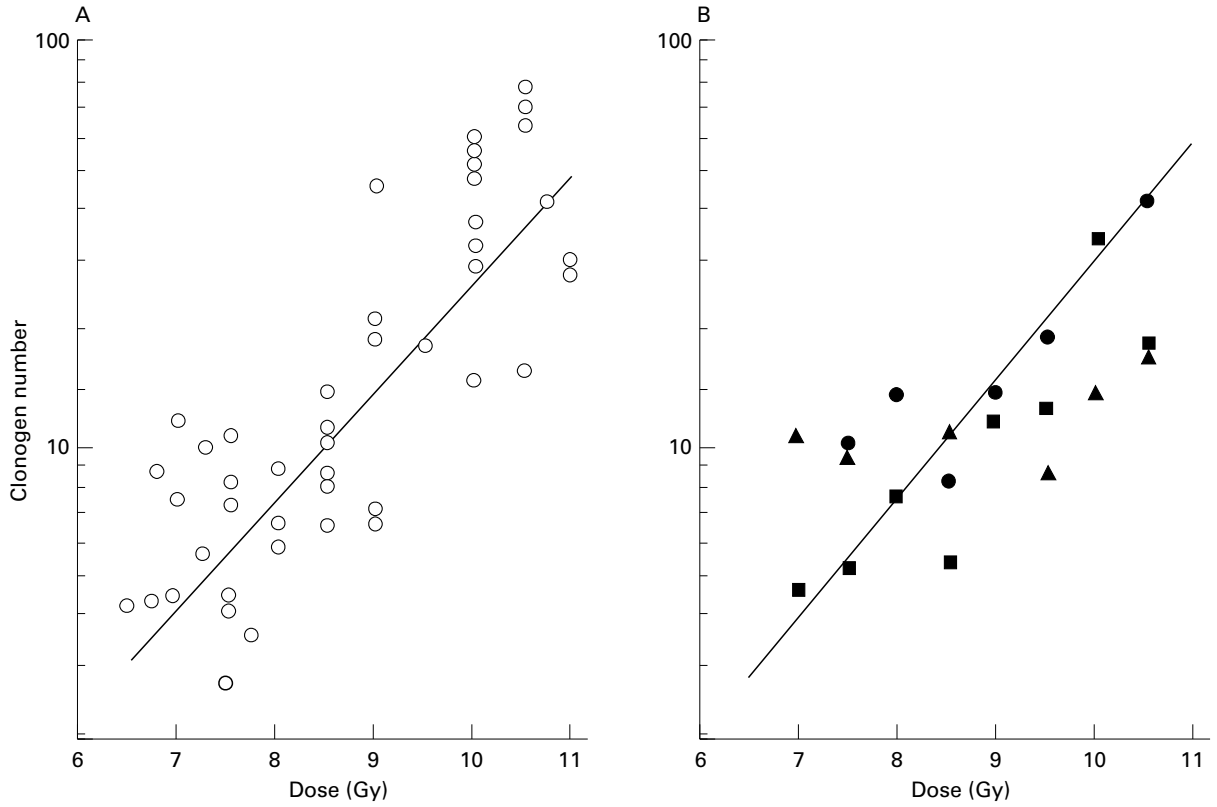

Figure 7. Estimates for the number of clonogenic cells per crypt using the two dose technique (Hendry 1979) (A) in the small intestine and (B) in the large intestine. ▲ Caecum, ■ Mid-colon, ● Rectum. The numbers increase as the dose used to assess the number is increased. Broadly, when the dose is below 9Gy the clonogenic number is about 6, while above 9Gy it is about 30. Redrawn from Cai *et al.* 1997b.

When higher clonal regeneration doses (greater than 9Gy) are used (greater cytotoxic stress) the estimate of clonogenic cell numbers increases to about 30–40 per crypt, see Figure 7. Thus, as the level of damage increases the crypts call upon (recruit) more and more clonogenic cells. These data therefore suggest that there may be a third and final tier of around 20 (16–24) clonogenic cells which are even more 'hardy' and are the final resource when the first two tiers of cells have been killed. Thus, there is a gradual loss of 'stemness' over two divisions and although a crypt may be 'using' 4–6 stem cells normally it has the ability to call upon about 36 cells (28–42) to ensure crypt survival. This is the total number of clonogenic cells per crypt and will comprise many of the cells around the crypt annulus at positions 2–7, leaving about 120 proliferating cells per crypt which appear to possess no clonogenic regenerative capacity. The existence of a high number of clonogenic cells, but few actual stem cells may partially explain the varying estimates of stem cell number published in the literature.

A recent series of experiments in our laboratory has extended these estimates of clonogenic cell numbers to the large intestine. Initially, the small intestinal micro-colony assay proved to be difficult to adapt to this tissue due to both the inherent radioresistance of the cells in the colon, and their slower cell cycle time. Thus, in order to distinguish between sterilized and surviving crypts the dose of irradiation must be greater and the assay time extended – a protocol that the mice cannot survive due to the radiosensitivity of other tissues (Withers & Mason 1974; Hamilton 1977; Hamilton 1979; Cai *et al.* 1997a). However, the use of lead shielding to restrict the irradiation to the abdomen overcame this problem (Cai *et al.* 1997b). These experiments indicated that, like the small intestine, there may be a small number (5–10) of stem cells with about 36 clonogenic cells in total and a similar dose dependence on the estimate of clonogenic numbers (see Figure 7).

If a crypt normally contains several functional stem cells the obvious question is whether each stem cell is producing a separate lineage, or all the stem cells are producing all the lineages. If each stem cell becomes a

committed cell for each lineage and serves to maintain that lineage one might expect transient changes in the relative numbers of each differentiated cell type as stem cell numbers occasionally change due to deletion or symmetrical division. This has not been reported.

It certainly appears that a single stem cell is *capable* of producing more than one lineage (i.e. is pluripotent). A number of experiments both *in vitro* and *in vivo* have indicated that when reduced to a single surviving cell the subsequently regenerated unit contains many lineages. For example, following a high dose of radiation that should theoretically reduce a crypt to a single stem cell, the regenerated crypts contain all the lineages (indicating that the surviving clonogenic stem cells are totipotent). Similarly, experiments with the mutated *Dolichos bifluros* and G6 PD crypts described above have shown mutated crypts containing all the lineages. Finally, isolated colonic tumour cells *in vitro* or cloned and grafted subcutaneously in mice have the ability to generate all the cell types (Kirkland 1988, Cox & Pierce 1982). These latter experiments indicate that a single colonic epithelial cell can generate all the cell types – but the caveat is that in these experiments the cell involved was transformed.

Each of these circumstantial observations strongly indicate a common origin, but do not prove conclusively that this is the case in a normal steady state crypt.

Spatial considerations for the stem cell population

As the localization of the stem cells in a crypt can be predicted, the behaviour of the cells at this position can be analysed and interpreted accordingly. For example, one can measure the cell cycle time at this position, or the incidence of apoptosis, and how different growth factors may affect either and possibly alter the number of functional stem cells.

The cell position in the small intestinal crypt most likely to contain the stem cells is the 4th position from the base of the crypt. An annulus of 16 cells are to be found at this position. It should be noted, however, that 'the 4th position' is an average for the situation both around an individual crypt and between different crypts. The most likely situation is that the stem cells are found immediately above the highest Paneth cell. This may fluctuate between a cell position as low as 2 or as high as 7 around an individual crypt. There may therefore be a undulating annulus of undifferentiated cells situated above the Paneth cells (and/or pre-Paneth cells) (Figure 8). Thus, if all 16 cells within this annulus were stem cells they may not be in direct contact, since in one upward ribbon of cells the stem cell may be located at position 2, whereas

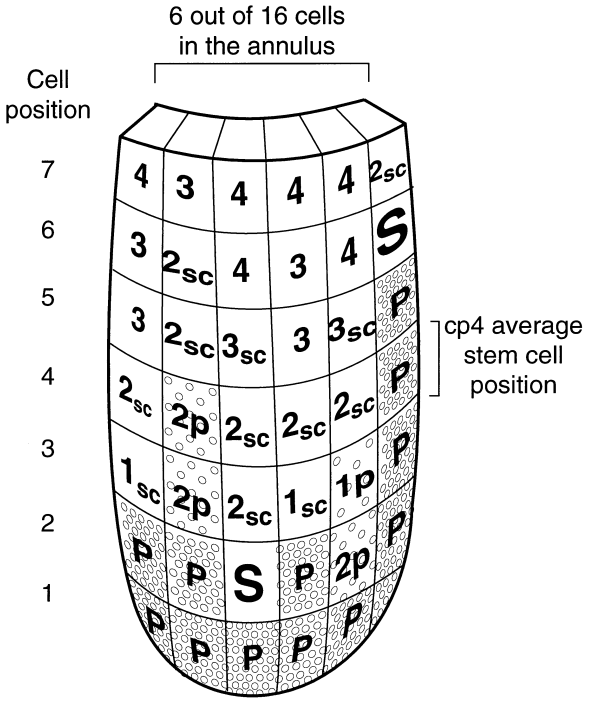

Figure 8. The actual stem cells are scattered above the mature Paneth cells (P) in an undulating annulus, at an *average* of cell position 4. The cartoon illustrates that the position of these stem cells may vary around the crypt circumference, and a stem cell may actually reside adjacent to 4th 'generation' cell – making it difficult to imagine how stem cell specific behaviour can be controlled by external factors. S, Stem cell; 1–4, 1st-4th generation daughter cells; Sc, those with a clonogenic potential; P, Paneth cell; 1P,2P, pre-Paneth cells.

in the adjacent upward ribbon the stem cell may be at position 7.

The current model for the stem cell population suggests that there may be upto 6 ultimate steady state stem cells, presumably spaced at fairly regular intervals around the undulating annulus of 16. When these cells divide they maintain their own numbers and produce a daughter that represents the first tier of the clonogenic compartment. The division of the stem cells is predominantly asymmetric but mathematical modelling suggests that perhaps 5% of the time the stem cells divide symmetrically producing an extra stem cell (Loeffler *et al.* 1993) which may have to be deleted by the spontaneous apoptosis process or by rapid differentiation. The 6 first-tier clonogenic cells are most likely to be located adjacent to the ultimate stem cells within the undulating annulus. These stem cells and the first tier clonogenic cells would therefore virtually fill this annulus with their 12 members. The remaining four positions would be occupied by 4 of the approximately 24 second-tier clonogenic cells. The

next annulus would then be completely filled with 16 further second-tier clonogenic cells.

This spatial configuration could account for the theoretical distribution of stem cells, clonogenic cells and apoptotic cells induced by 1Gy of radiation and would provide a stable geometric configuration for the steady state production within the crypt.

The observations suggest that the number of stem cells is tightly regulated with any extra stem cells being removed by spontaneous apoptosis and any reduction in number being compensated for by changes in proliferation in the remaining cells within the annulus (Potten 1995a, b). The difficulty one encounters with this spatial model is how to explain the stem cell homeostatic mechanisms. How do stem cells know that there should be six and how do they recognize that there are seven or five? This is particularly difficult since these cells would not appear to be in direct contact (see Figure 8). The implications are that the ultimate stem cells generate a field, or shell of stemness and it is the overall concentration of this factor that determines whether a cell dies or other cells enter a stimulated round of division.

Stem cell cycle times

In the mouse the cell cycle time of the actual stem cells has been estimated using a variety of approaches resulting in estimates of between 12 and 32 hs; up to twice as long as the cells higher up the crypt (Potten 1986). The presence of more slowly cycling cells, located at about position 4, can be demonstrated by appropriate labelling studies followed by grain dilution analysis. Two or three doses of tritiated thymidine spaced 6 h apart will label essentially all the proliferating cells in the crypt including the more slowly cycling putative stem cells. If autoradiographs are prepared from tissue fixed 2, 4, 6,and 8 days after labelling the bulk of the rapidly proliferating cells of the crypt will have diluted the label to subthreshold detection levels. However, while the label in the more slowly cycling stem cells will also have diluted, this will be less rapid and at an appropriate time these cells can therefore still be recognized by the presence of label. This can be enhanced by administering the labelling protocol immediately after a dose of 8–10 Gy of radiation. Under these circumstances it is also possible that the retention of label in the stem cells (which in this circumstance are dividing symmetrically to increase the stem cell number) may be enhanced by the selective retention of labelled DNA strands (Cairns 1975; Potten *et al.* 1978).

In humans, the length of the stem cell cycle period is less well defined, but is thought to be between four and eight times longer (Wright & Alison 1984; Potten 1992). It is possible that the slower cell cycle time in stem cells generates fewer opportunities for errors in DNA synthesis and may allow extra time for the repair of those that do occur, i.e. allows for genetic housekeeping. Interestingly, such cycle times over the lifetime of mouse and man imply 1000–5000 cell divisions, respectively, i.e. stem cells have a high degree of self maintenance.

Factors controlling proliferation

The conditions at the stem cell position in the crypts, i.e the growth factors, cytokines, and extracellular matrix (ECM) molecules present, must be optimum for stem cell maintenance and homeostasis – and define the stem cell 'niche'. For example, the niche factors may cause the longer stem cell cycle time, influence self-maintenance properties, prevent differentiation, and prevent the upward migration of stem cells by increasing their attachment properties. In addition the stem cells themselves may express specific combinations of growth factor receptors and integrins in response to the local extracellular environment. Hence, the regulation of this region may involve a different cocktail of factors than those that regulate the proliferating daughter cells.

Exactly how these factors interact, or indeed what they are, is not understood, but their effects are gradually being dissected, in order to build up a more complete picture of cell cycle regulation up the crypt-villus axis. For example, immunohistochemical staining of intestinal tissue has revealed the presence (and therefore implied a role for) a number of growth factors and their receptors. Similarly, growth factor or extracellular matrix effects on a number of cloned intestinal epithelial cell lines have been assessed. However, to date no single growth/ECM factor or receptor has been shown to be exclusively localized in the stem cell region of the crypt.

Growth factors

One of the most widely investigated growth factors is epidermal growth factor (EGF), and its related family members. EGF is abundant along the upper gastrointestinal tract suggesting that it plays a role in the regulation of this tissue. However, it is difficult to envisage subtle stem cell regulation by such an abundant factor, and lumenal (oral) administration of this factor has no effect on cell proliferation (Bamba *et al.* 1993; Ribbons *et al.* 1994; Playford & Wright 1996). EGF appears to be more important for increasing proliferation and migration in wounded tissue (leading to wound restitution and repair) than on the regulation of normal homeostasis

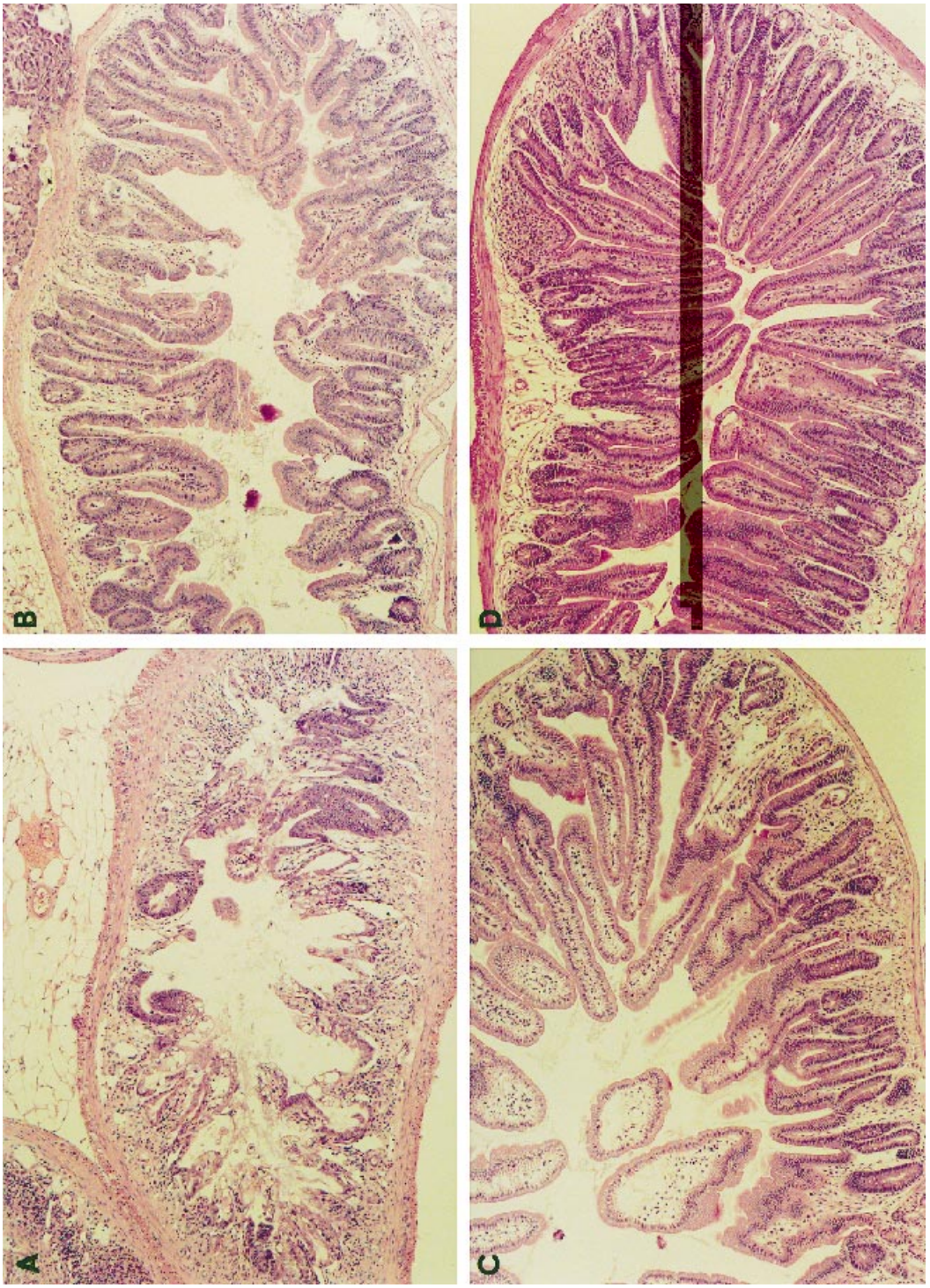

Figure 9. Small intestinal morphology following 14.5Gy irradiation. After 8 days the mucosa is totally destroyed and the lamina propria is exposed (A). Pre-treatment with TGFβ-3 protects the stem cells, and hence the mucosa from such acute damage (B, also day 8). Many more surviving crypts can be seen. Architectural restoration can be seen on day 14 in the TGFβ treated group (C) and a fully repaired tissue, can be seen after 30 days (D). This day 30 morphology appears identical to the unirradiated control tissue.

(Konturek *et al*. 1988; Saxena *et al*. 1992; Potten *et al*. 1995). Indeed, following irradiation EGF and TGF-α appear to be up-regulated, whereas TGF-β expression levels are reduced (Ruifrok *et al*. 1997). Combined with the data indicating that members of the EGF family are important for crypt development in neonatal animals, these observations suggest that although present in the lumen these factors only have a mitogenic/motogenic role in damaged, regenerating or growing tissue.

Most of the other members of the EGF family are recognized by the same receptor but may have different binding affinities and erbB signalling partners and, therefore, have slightly different roles. This may also be a means by which more subtle clonogenic cell specific responses can be elicited. For example, there is some evidence that they regulate apoptosis in certain situations, including damaged gastrointestinal epithelium, and may protect the mucosa from certain cytotoxic effects (Romano *et al*. 1992; Booth & Potten 1996 and reviewed by Reddy 1996)

The insulin-like growth factors (IGF-1 and IGF-2) have also been shown to regulate crypt cell proliferation (Vanderhoof *et al*. 1992; Park *et al*. 1992; Potten *et al*. 1995; Conteas *et al*. 1987; Booth *et al*. 1995). However, understanding cell cycle regulation by the IGFs is complicated by the fact that a number of IGF binding proteins control the local availability of the factors and that IGF receptor expression has been shown to be regulated by the extracellular matrix (ECM) composition – and so changes in ECM components along the crypt (e.g.in basal vs. mid-crypt regions) may alter the ability of IGF to induce mitogenesis (Benya *et al*. 1993). However, to-date there is no evidence that these factors alone can modulate stem cell function.

Almost all the interest to-date on negative regulators of epithelial proliferation has focused on the TGF-β family of factors and their receptors. The TGFβ's have been shown to inhibit epithelial proliferation from stomach to colon inclusive (Lamprecht *et al*. 1989; Migdalska *et al*. 1991; Potten *et al*. 1995). This has also been reproduced *in vitro* (Barnard *et al*. 1989; Booth *et al*. 1995). The role of the TGFβ's in regulating the gastrointestinal epithelium is still controversial with two, not necessarily exclusive, options. The first set of evidence indicates that these factors are responsible for the upper crypt and villus cells' exit from the cell cycle and/or entry into terminal differentiation – hence increased expression of factor and receptor has been found in these mature cells, and the factor has been demonstrated to induce the differentiated phenotypes *in vitro* (Kurokowa *et al*. 1987; Barnard *et al*. 1989, 1993; Avery *et al*. 1993). The alternative view is that TGF-β regulates crypt cell output in the proliferative zone of the crypt, including the stem cells. This is supported by papers reporting increased expression in this region of the tissue (Koyama & Podolsky 1989). Protracted *in vivo* administration has been shown to reduce crypt cell proliferation and possibly alter stem cell cycling (Potten *et al*. 1995). Furthermore, stem cells appear to be protected from cytotoxic insult (as measured by the clonal regeneration assay) by TGF-β pretreatment (Potten *et al*. 1997), Figure 9.

A number of interleukins also appear to affect the regulation of the gastrointestinal epithelium. For example, the IL-2 receptor has been found on epithelial cells, and mice lacking IL-2 exhibit ulcerative colitis (Ciacci *et al*. 1993; Sadlack *et al*. 1993). However, it is unlikely that this represents a stem or proliferative cell response and is purely immunological, although IL-2 has been shown to stimulate $\delta\gamma$T cells to produce keratinocyte growth factor (KGF), a mitogen that may also protect the gut stem cells from cytotoxic insult (Boismenu & Havran 1994, Zeeh *et al*. 1996; Farrell *et al*., unpublished observation). IL-11 and IL-6 and therefore by implication the related factors that use the same signal transducer as IL-6) have also been reported to induce KGF expression (Chedid *et al*. 1994; Brauchle *et al.* 1994). In addition to stimulating DNA synthesiz, KGF appears to cause increased crypt length and induce crypt production via bifurcation (fision) which could be a consequence of increased stem cell number (Housley *et al*. 1994; Finch *et al*. 1996). KGF has also been reported to have specific lineage effects, increasing the number of goblet cells per crypt (Housley *et al*. 1994).

Direct effects of interleukins on epithelial cells *in vitro* have been demonstrated by IL-4 (which is stimulatory) and IL-6 and IL-11 (which are inhibitory) (Booth & Potten 1995; Peterson *et al*. 1996; McGee & Vitkus 1996). There is also increasing evidence for an interaction between the interleukins and conventional growth factors as a mechanism of controlling epithelial, and possibly stem cell, proliferation. For example, TGFβ and IL-11 appear to behave similarly *in vitro* (Booth & Potten 1995) and it has been shown that TGFβ can induce IL-6 and IL-11 production (McGee *et al*. 1992, 1993; Elias *et al*. 1994).

In vivo experiments using IL-11 have also demonstrated crypt stem cell regulatory effects. This was observed as an increased crypt survival (i.e. stem cell survival) following acute irradiation when mice were pretreated with the factor (Du *et al*. 1994; Orazi *et al*. 1996; Potten 1996). IL-11 has also been reported to modulate stem cell responses to irradiation, sensitising or protecting depending upon the dosing protocol (Hancock *et al*. 1991). This may be related to the

observation that IL-11 can stimulate IL-6 or KGF production (McGee *et al.* 1993; Chedid *et al.* 1994; Brauchle *et al.* 1994).

Interestingly, neither IL-6 nor IL-11 appear to induce measurable effects on DNA synthesis or apoptosis, possibly implying that the *in vivo* effect may be very subtle, and acting upon a very small population of cells. One interesting possibility that is suggested by other tissues is that such cytokines may alter epithelial $\alpha 5 \beta 1$ integrin expression (the fibronectin receptor) which in turn increases adhesion and survival by increasing bcl-2 expression (Ohashi *et al.* 1995; Zhang *et al.* 1995).

Adhesion molecules

The ECM underlying the epithelium is known to contain a number of molecules, most notably, E-cadherin (uvomorulin), tenascin, laminin, fibronectin, collagen IV, nidogen and perlecan. The ability of a stem cell to remain anchored at the crypt base, or a daughter cell to move away from this position, must result from increased or decreased affinity for one or more of these molecules. This altered affinity may be due to changed expression/availability levels of either the ECM molecule or its cellular receptors (integrins). Interestingly, there is increasing evidence that adhesion molecules and growth factors may regulate each other's expression. Furthermore, integrins can also directly regulate the cell cycle (Juliano & Varner 1993). Increased anchorage due to overexpression of specific integrins has been demonstrated for keratinocyte stem cells (Jones *et al.* 1995), but currently there are no suitable assay systems available to demonstrate such effects in the gut. However, there is accumulating circumstantial evidence that ECM molecules alter enterocyte adhesion and migration. For example, fibronectin (adhesive) is more abundant in the crypt, whereas tenascin (less adhesive) is more abundant on the villus (Figure 10), which correlates with the increased migration rate observed as cells move up the villus (Kaur & Potten 1986; Beaulieu 1992). Fibronectin has also been shown to be overexpressed by proliferating intestinal epithelial cells, and to inhibit differentiation in keratinocytes, which is consistent with its localization and suggests a function within the crypt (Adams & Watt 1989; Vachon *et al.* 1995). The different laminin isoforms may also have a distinct crypt-villus distribution, whereas E-cadherin and collagen appear to be universally distributed (Beaulieu 1992; Beaulieu & Vachon 1994), Figure 10. A role for E-cadherin in regulating epithelial organization, and hence function, has been deduced from studies using chimeric-transgenic mice techniques (Hermiston *et al.* 1993; Hermiston & Gordon 1995a, b). This model

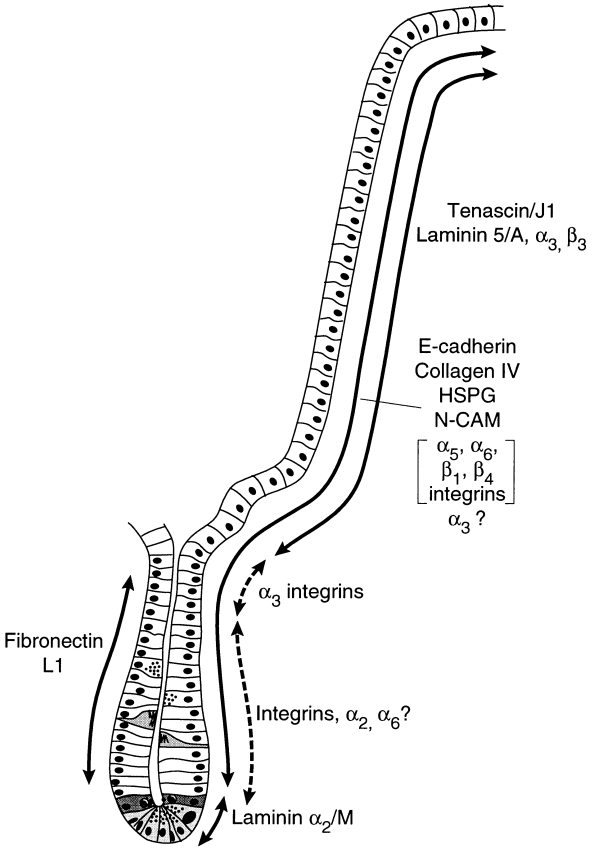

Figure 10. Cartoon illustrating the spatial expression of integrin and extracellular matrix adhesion molecules in the small intestine [data (in some cases preliminary) taken from published literature]. Conflicting data have been published for the $\alpha 3$ and $\alpha 6$ integrins, hence the alternative expression pattern is denoted by '?'

system is derived from experiments in which embryonic stem (ES) cells (129/Sv origin) were transfected with a reporter gene linked to transcriptional regulatory elements from one of the fatty acid binding protein genes described earlier. These were subsequently introduced into host C57BL/6 blastocysts. Thus, at the borders of ES cell-derived and host blastocyst-derived epithelium, there were villi supplied by ES cell and host blastocyst derived crypts. Such villi could be readily identified in whole mount preparations by staining with α-L-fucose specific *Ulex europaeus* agglutinin type 1 (UEA-1) lectin, which recognizes a cell-surface carbohydrate polymorphism between the inbred strains used to generate the chimeric animals.

This system allowed the effect of a single gene product to be assessed within a single villus, while the band of blastocyst-derived epithelium within the same villus acted as an internal control in an identical microenvironment. The

system also has the advantages of being able to detect subtle phenotypes and of allowing the study of gene products which have toxic effects on the gut (by controlling the degree of chimerism by the number of ES cells originally injected into the blastocyst).

This technique was successfully adapted to study the effects of E-cadherin in the mouse intestine. Hermiston & Gordon (1995b) initially expressed a dominant negative N-cadherin mutant (NCADΔ) under the control of the *I-FABP*$^{-1178\ \text{to}\ +28}$ promoter, which is only expressed in enterocytes on the small intestinal villus. The resultant villi in these chimeric mice suggested that NCADΔ disrupts cell-cell and cell-matrix contacts. There was an increased rate of migration of enterocytes along the crypt-villus axis, a loss of their differentiated phenotype, and precocious entry into the death program. The same authors subsequently expressed the same NCADΔ on a *L-FABP*$^{-596\ \text{to}\ +21}$ promoter to generate chimeric mice in which NCADΔ is expressed along the entire crypt-villus axis (Hermiston & Gordon 1995c). These animals developed inflammatory bowel disease with similar histology to Crohn's disease and also intestinal adenomas. Crucially, these lesions only occurred in epithelium derived from the transfected ES cells. These experiments have suggested that cadherins play an important role in controlling the fate of small intestinal stem cells, since tissue expressing mutant protein shows an altered phenotype.

Regulation of cell number by apoptosis

Apoptosis is a form of programmed cell death with particular morphological characteristics and is crucial for homeostasis in the normal intestinal epithelium. It is responsible for the removal of excess but otherwise healthy cells and also the removal of cells which have sustained damage.

Morphologically apoptotic cells can be detected in cross sections of intestinal epithelium by light and electron microscopy. The incidence of apoptotic cells can be related to their position along the long axis of the crypt and statistically valid results can be obtained by counting 200–300 well orientated half crypts from groups of 4–6 mice (Ijiri & Potten 1983, 1985; Li *et al*. 1992; Merritt *et al*. 1995, 1996) (see Figure 1). This method has been compared with whole mount techniques and it has been shown that 60–80% of apoptotic events can be detected by the counting of sections (Merritt *et al*. 1996; Potten & Grant, unpublished observation). An alternative method of analysing apoptosis is an *in situ* Tdt-mediated dUTP-biotin nick end labelling technique (TUNEL) as first described by Gavrieli *et al*. (1992). This method is based upon the internucleosomal DNA cleavage which occurs during apoptosis. Unfortunately, this technique is prone to false positive and false negative results when compared with morphological techniques, though modifications in the protocol (Hall *et al*. 1994; Merritt *et al*. 1995, 1996) have reduced the frequency of these. The technique also lacks the ability to distinguish between apoptotically cleaved DNA and DNA fragments generated by other mechanisms.

Occasional apoptotic cells can be observed in the intestinal crypts of healthy mice and man, henceforth termed *spontaneous apoptosis*. In the normal murine small intestine approximately one apoptotic cell is seen in every 5th longitudinal crypt section, implying that less than 1% of crypt cells are apoptotic at any particular time. However, this apoptosis occurs predominantly at cell positions 4–6 (the stem cell zone), suggesting that upto 5–10% of stem cells are undergoing apoptosis at any one time (Potten 1977, 1992). A circadian rhythm in this apoptosis occurs with maximum levels occurring in the early hours of the morning, just after the middle of the dark period of the 12 h light/dark cycle (Potten *et al*. 1977). It has been postulated that this spontaneous apoptosis in the stem cell zone helps to maintain stem cell homeostasis, which is important because one additional stem cell could lead to an extra 60–120 cells per crypt. Similar spontaneous apoptosis can be observed in the mouse colon, though the frequency is less and the apoptosis is not clustered around the stem cell zone (which in this case is believed to be located at the crypt base).

The effects of expression of the apoptosis-controlling genes *p53* and *bcl-2* upon this spontaneous apoptosis have also been studied using homozygously null mice. Homozygously *p53* null mice (Donehower *et al*. 1992) showed similar levels of spontaneous apoptosis in the intestinal epithelium compared with their wild type counterparts (Clarke *et al*. 1994; Merritt *et al*. 1994). Homozygously *bcl-2* null mice (Nakayama *et al*. 1993) showed similar levels of spontaneous apoptosis in the small intestinal crypts as their wild-type counterparts, but in the colon there were elevated levels of spontaneous apoptosis which was concentrated at cell positions 1–2 at the base of the crypt, where bcl-2 protein is expressed and where the colonic stem cells are believed to be located (Merritt *et al*. 1995). Our preliminary analysis of homozygously *bax* null mice (Knudson *et al*. 1995) has not revealed any alteration in spontaneous apoptosis in these animals.

There has been much debate concerning whether apoptosis also occurs at the villus tips. TUNEL positive cells can be found at this location (Gavrieli *et al*. 1992; Hall *et al*. 1994), but initial electron-microscopic studies found only very rare apoptotic cells (Potten & Allen 1977;

Kerr *et al*. 1987). Han *et al*. (1993) and Iwanaga *et al*. (1993) have studied guinea pig intestinal epithelium and Shibahara *et al*. (1995) studied human small intestinal epithelium electron microscopically and presented evidence for a specialized form of apoptosis at this site. Furthermore, *Bax*, a cell death gene, is expressed at the villus tip and on the intercrypt table region in the mid-colon (Wilson & Potten 1997), supporting the hypothesis that cell loss occurs via a form of apoptosis at these sites.

Apoptosis can be induced in the murine intestinal epithelium by γ-radiation and a wide variety of cytotoxic agents. In the small intestine *radiation-induced apoptosis* occurs predominantly in the stem cell region at doses as low as 1–5cGy (Potten 1977). 1Gy induces about 6 apoptotic cells per small intestinal crypt, predominantly at the stem cell position. This number represents a plateau, since higher radiation doses have no dramatic additional effect. Radiation-induced apoptosis also occurs in the murine colon, but as with spontaneous apoptosis, this is not consistently centred at the stem cell positions and occurs at a lower level per unit of dose. The plateau in apoptotic yield in the mid-colon occurs with radiation doses above 6–8Gy (Potten 1992, 1995b; Potten and Grant, unpublished observation). Ijiri & Potten (1983, 1985) studied the apoptotic response of the murine small intestine to 15 cytotoxic agents; each agent had selectivity for cells of particular hierarchical status along the crypt-villus axis. Two subsequent papers studied the response of murine small and large intestine to a series of four carcinogens (Li *et al*. 1992; Potten *et al*. 1992). Together these experiments indicated that all the cell positions in the crypt hierarchy are capable of undergoing apoptosis under the correct conditions, but the mechanism and significance of the selectivity has not been established. Lee (1993, 1994) has also demonstrated an increase in apoptotic cells in the human intestine following 5-fluorouracil administration.

Thus, both spontaneous and radiation induced apoptosis in the small intestine together with the apoptosis induced by isopropylmethane sulphonate, bleomycin and adriamycin as well as the apoptosis induced by 4 chemical mutagens all appear to be associated with cells that occur at the stem cell position in mice (Ijiri & Potten 1983, 1985; Li *et al*. 1992). Hence, this form of cell death after these agents appears to be a property associated with the stem cells in this tissue. The apoptosis related genes involved in regulating these processes, and the expression of their proteins, can therefore be regarded as a form of marker characterizing small intestinal stem cells.

There have been reports that different mouse strains show different apoptotic responses to radiation with C3H strains showing lower levels of jejunal crypt cell apoptosis than C57BL/6 J mice (Weil *et al*. 1996). Recent preliminary experiments in our laboratory have shown little strain differences in both spontaneous and radiation induced apoptosis (Potten, Grant, O'Shea, unpublished data). However, we have demonstrated differences in small intestinal apoptosis between DBA/2 and Balb/c strains following intraperitoneal administration of the thymidylate synthase inhibitor Tomudex (Pritchard, unpublished observation).

The genetic control of the damage-induced apoptosis described above has also been studied using immunohistochemistry and transgenic mice. The cell positional distribution of p53 protein expression correlated strongly with the position of apoptotic cells in the murine intestine following both radiation (Merritt *et al*. 1994) and 5-fluorouracil (Pritchard *et al*. 1997), but occurred predominantly in non-apoptotic cells. Furthermore, homozygously *p53* null mice showed absence of the normal intestinal apoptosis in the 3–4-hour period following radiation (Clarke *et al*. 1994; Merritt *et al*. 1994) and in the 24-hour period following 5-fluorouracil administration (Pritchard *et al*. 1997). However, these mice showed apoptotic cells 24 h following 8Gy radiation (but not 1Gy). The morphology of these apoptotic cells suggested that they may have arisen as a result of aberrant mitosis (Merritt *et al*. 1997). Homozygously *bcl-2* null mice showed elevated levels of apoptosis specifically at the stem cell zone at the base of colonic crypts following γ-radiation – the site of bcl-2 protein expression in wild-type mice (Merritt *et al*. 1995). Following the alternative stimulus of 5-fluorouracil, homozygously *bcl-2* null mice again showed increased levels of apoptosis at this site 4.5 h after drug administration, although by 24 h levels were equivalent in wild-type and null mice, suggesting that *bcl-2* may act to delay apoptosis rather than preventing it completely, or that the apoptosis seen after 24 hs is *bcl-2* independent (Pritchard *et al*. manuscript in preparation). The effects of other *bcl-2* family members have not yet been studied in this context, but the genetic control is likely to be much more complex than currently understood.

Apoptosis therefore controls cell number in the crypt compartment both in the steady state and following damage. This apoptotic response, which is influenced by the expression of genes such as p53 and bcl-2 family members, is summarized in Figure 11.

Implications of stem cells and apoptosis

Stem cells and their deletion by apoptosis are likely to have important implications regarding the development, treatment and prevention of gastrointestinal tract cancer.

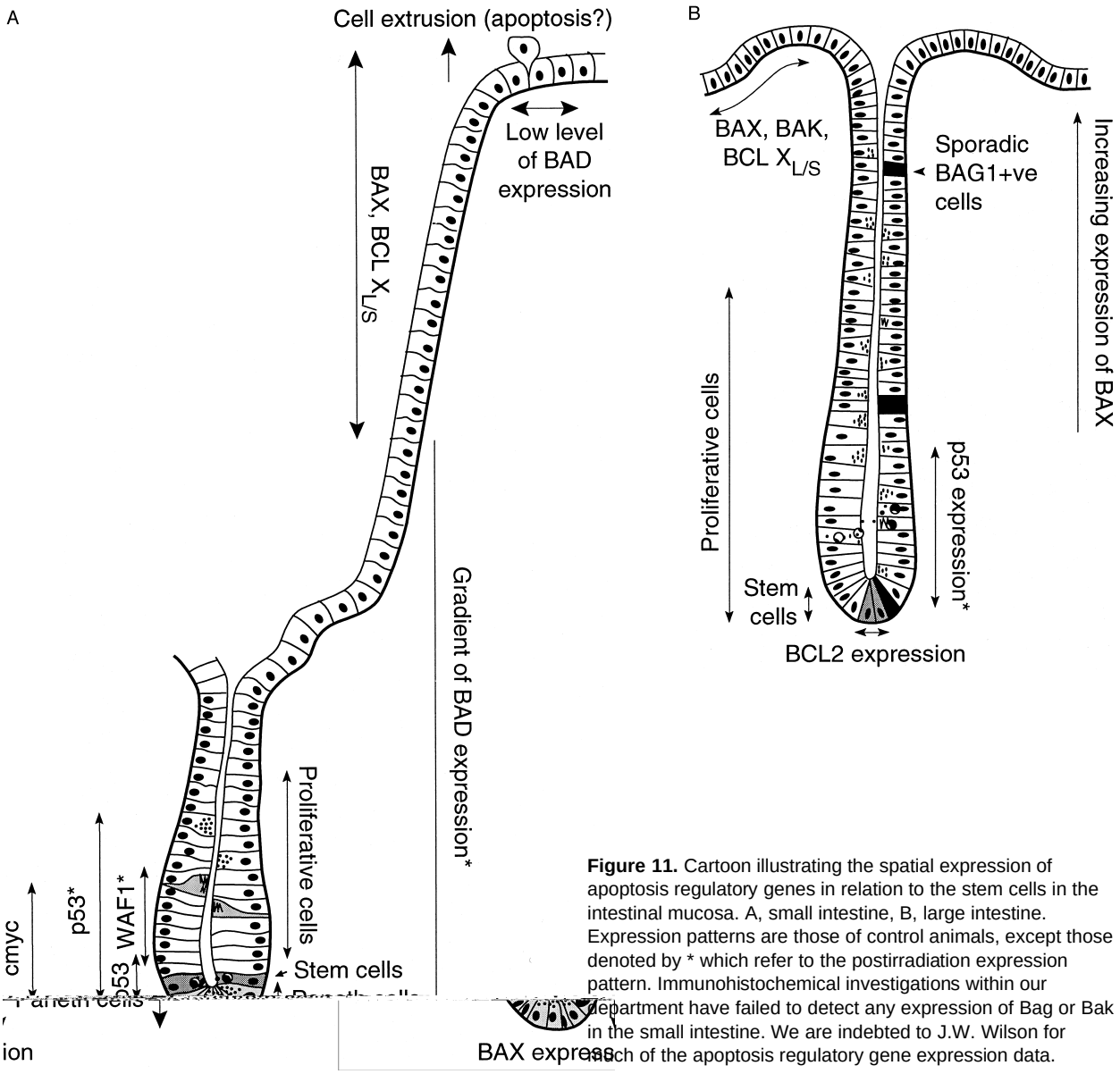

Figure 11. Cartoon illustrating the spatial expression of apoptosis regulatory genes in relation to the stem cells in the intestinal mucosa. A, small intestine, B, large intestine. Expression patterns are those of control animals, except those denoted by * which refer to the postirradiation expression pattern. Immunohistochemical investigations within our department have failed to detect any expression of Bag or Bak in the small intestine. We are indebted to J.W. Wilson for much of the apoptosis regulatory gene expression data.

Colorectal neoplasms are believed to arise from mutations within the pluripotent stem cell, though a recent paper has reported the polyclonal origin of colonic adenomas in an XO/XY patient with familial adenomatous polyposis (Novelli *et al*. 1996). Fearon & Vogelstein (1990) proposed that sequential mutations occurred in key genes such as *APC, K-ras, DCC* and *p53*. *p53* inactivation is a proposed late event in this cascade because immunohistochemical analysis has shown mutant *p53* in late stage tumours having a poorer prognosis (Baker *et al*. 1990; Purdie *et al*. 1991). It has been suggested that the strong *p53* dependence of γ-radiation-induced apoptosis in small intestinal stem cells contributes to the low incidence of cancer at this site (Merritt *et al*. 1994). However, the situation is likely to be more complex since mice bred by crossing *p53*-null and *Min* show no change in the spectrum of intestinal tumours, suggesting that *p53* is not involved in the early stages of intestinal tumourigenesis (Clarke *et al*. 1995).

Evidence suggests that the antiapoptotic gene *bcl-2* is also involved in colorectal tumourigenesis. The product of this gene can be detected immunohistochemically, especially in early colonic adenomas (Bosari *et al*. 1995; Bronner *et al*. 1995; Hague *et al*. 1994; Sinicrope *et al*.

1995; Watson *et al*. 1996). There also appears to be a reciprocal relationship between bcl-2 and p53 expression (Watson *et al*. 1996). The increased levels of spontaneous and radiation induced apoptosis at the stem cell positions at the base of colonic crypts in *bcl-2* knockout mice (Merritt *et al*. 1995) suggests that *bcl-2* normally serves to protect the vulnerable colonic stem cells from toxin-induced apoptosis, but it may also allow mutated genes to survive which may lead to carcinogenesis.

Cytotoxic agents induce apoptosis in the murine intestinal epithelium (Ijiri & Potten 1983, 1985). It has been suggested that differential susceptibility to apoptosis may reflect tumour responsiveness to therapy (Dive & Hickman 1991). Different cytotoxic drugs target different cell positions within the crypt hierarchy, but the implications of this observation have not been established. We have recently demonstrated that *p53*-null mice, which have greatly reduced levels of intestinal apoptosis following 5-fluorouracil also have reduced levels of histological gut damage following consecutive doses of the drug (Pritchard *et al*., unpublished observation). This suggests that the expression of apoptosis controlling genes influences the response of normal intestine to cytotoxic agents, and one could speculate that tumours would behave similarly.

Current therapy for advanced colorectal cancer has limited benefits. Hence preventive strategies are important. Current interest has focused particularly on dietary measures and nonsteroidal anti-inflammatory drugs (NSAIDs). Short chain fatty acids, such as butyrate, which can be formed from the bacterial fermentation of dietary complex carbohydrates have been shown to induce apoptosis in a number of colon adenoma and carcinoma cell lines (Hague *et al*. 1993, 1995; Heerdt *et al*. 1994). It is therefore possible that these agents cause apoptosis following mutation in colonic stem cells. NSAIDs are chemoprotective in colorectal cancer (reviewed in DuBois 1995; Eberhart & DuBois 1995). They have been shown to induce apoptosis in cell lines (Piazza *et al*. 1995; Shiff *et al*. 1995) in a *p53*-independent manner and also in the normal colonic mucosa of patients with familial adenomatous polyposis (Pasricha *et al*. 1995). Evidence is accumulating that this effect is mediated via the inhibition of cyclooxygenase-2 (*COX-2*), since mice formed by crossing *COX-2* nulls with $Apc^{\Delta 716}$ showed a reduction in the number and size of intestinal polyps (Oshima *et al*. 1996). Furthermore, Tsuji & DuBois (1995) demonstrated that COX-2 overexpression inhibited apoptosis in RIE-1 cells by upregulating *bcl-2.*

The intestinal stem cell is therefore not only involved in tumourigenesis, but also influences the drug responses of the tissue. An ability to target agents to the intestinal stem cell would enable effective tumour chemoprevention, while agents which could limit damage to stem cells would improve the therapeutic index of chemotherapeutic drugs.

Conclusion

Clearly the main limitation to the current study of gastrointestinal stem cells is the lack of a suitable marker. Although many attempts have been made to identify such a molecule/gene, to-date all have failed. It is possible, however, that this may not be due to lack of technical ability, but due to the intrinsic nature of the stem cells themselves. These cells are, by definition, undifferentiated and therefore may be characterized by the *lack* of expression of particular genes. Such a 'fishing' exercise, already difficult due to the rarity of such cells in the mucosa, may therefore turn out to be impossible. However, this review has illustrated the striking advances that can be made using protocols that either enrich or perturb the behaviour of such cells and then observing the consequences of such an action. Many questions still remain unresolved – but continued application of new technologies should help us to understand these cells, and also rapidly convert any findings into clinical applications.

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