Bone marrow cells contribute to tissue regeneration in the intestine and skin

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ABSTRACT

Adult bone marrow contains progenitor cells that can extricate themselves from their bone marrow cavity niche, and engraft within foreign tissues, whereupon they produce specific differentiated adult lineages. Bone marrow engraftment is upregulated with increasing regenerative pressure, which has triggered speculation as to the therapeutic potential of bone marrow cells. In this thesis, I describe for the first time, that transplanted adult bone marrow cells engraft within the intestines of mice and humans and give rise to a substantial proportion of the mesenchymal cells in the lamina propria, namely, the intestinal subepithelial myofibroblasts. I show that bone marrow contribution to intestinal myofibroblasts is significantly enhanced in a mouse model of colitis. Furthermore, I show that bone marrow cells form multiple vascular lineages in the mouse colon, with an enhanced propensity in colitis. These results have implications for the use of bone marrow in the treatment of inflammatory bowel disease, either by directly producing mesenchymal and vascular lineages, and/or possibly *via* the indirect regulation of intestinal epithelial cells.

I also report that transplanted bone marrow cells engraft into the skin and contribute to multiple epidermal lineages. These bone marrow-derived cells are commonly located within postulated epidermal stem cell niches, and can express putative epidermal stem cell markers. Bone marrow cell engraftment was significantly enhanced in the wounded epidermis, and bone marrow-derived epidermal cells can proliferate *in vivo*, and form colonies *in vitro.* I found no evidence that bone marrow cells fuse with indigenous epidermal cells to form heterokaryons.

These results highlight the potential of bone marrow cells in the treatment of diseases of the gastrointestinal tract and skin.

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Thank you to my sisters, Kirsteen and Pamela, my best friends. And finally, thank you to Mum and Dad, for everything. This thesis is dedicated to you both. And now I promise to get a proper job.

TABLE OF CONTENTS

TABLE OF FIGURES

CHAPTER IV

CHAPTER V

LIST OF TABLES

CHAPTER I

General Introduction

1.1) Adult stem cells

Adult stem cells are primitive, morphologically indistinct cells that lack markers of lineage commitment, and are thus generally defined by their functional characteristics. All adult stem cells, referred to as stem cells herein, share two main properties. Firstly, all stem cells can undergo self-renewal by dividing to produce an identical daughter cell, thereby forming and maintaining the foundations of a tissue and ensuring stem cell longevity within the tissue. Indeed, stem cells are often defined by their capacity for clonogenic self-renewal throughout the lifetime of their host. Secondly, stem cells can divide to produce daughter cells that evolve, or "differentiate", to form the entire specialised adult cell repertoire within their tissue of origin. This ability to produce cells of more than one lineage is known as “multipotentiality”. Stem cells divide to produce a partly-differentiated intermediate cell, known as a "transit amplifying" cell. These cells are committed to the formation of terminally-differentiated adult cell lineages, typically believed to be confined to a particular cellular pathway, although emerging data indicates that the classical pathways of stem cell differentiation are not as definitive as previously believed (section 1.13).

Fundamentally, stem cells preserve the steady-state functioning of a tissue and maintain homeostasis by replenishing cells that are lost due to damage or disease. In tissues with a rapid cell turnover rate, such as the gastrointestinal tract and the skin, cells are constantly being shed and replaced by the differentiating cells beneath them, and concurrently, the

rate of stem cell production of transit amplifying cells is higher in these tissues. Likewise, the rate of stem cell division fluctuates in response to regenerative demand dictated by tissue disease and injury.

The onset of neoplastic change within a tissue generally occupies a significant time span, as a number of mutations must accumulate within a cell before neoplasia is invoked. Therefore in tissues such as the skin and gastrointestinal tract, wherein the differentiated adult cells are distinctly short-lived, it is likely that the stem cells are the carcinogen target cells, due to their prolonged existence within these tissues. The gradual accumulation of mutations within a stem cell is believed to lead to the formation of a "cancer stem cell", which divides to produce a clone of mutated daughter cells and leads to tumour formation (Al-Hajj et al., 2004; Park et al., 2004; Reya et al., 2001). Elucidation of the genetic adaptations that lead to the formation of cancer stem cells will pave the way to a clearer understanding of the processes of tissue disease and repair, and hopefully the development of effective future therapies.

1.2) The stem cell niche

Stem cells are located within a specialised regulatory microenvironment, or niche, which provides an optimal milieu for stem cell survival and function. The "stem cell niche" hypothesis was defined by Schofield in 1978 (Schofield, 1978), and proposes that specific signals from the niche cause the stem cells to undergo self-renewing divisions, thereby preventing their maturation. When stem cell progeny migrate outwith the niche they differentiate to become transit-amplifying cells, demonstrating that stem cells may be regulated by their environment, rather than by intrinsic programming.

Due to their indistinct phenotype, stem cells within most mammalian adult tissues have proven difficult to identify, and consequently, characterisation of the components comprising the stem cell niche that govern and support the stem cells is equally arduous. The mammalian stem cell niche is thought to be formed by the stromal cells and their secreted extracellular matrix (ECM), which surround the stem cells. Due to the hierarchical migratory nature of differentiating transit amplifying cells, the stem cell niche is most likely to be situated at the origin of cellular flux, as associations between the stem cells and the cells of the niche are vital for the maintenance of tissue turnover. The "conceptual" stem cell niche should consist of three primary components: the localised signalling cells and their secreted extracellular matrix factors, the cells which comprise the niche; the target range influenced by the signalling factors of the niche, the expanse governed by the so-called niche; and the target of the signalling factors, the stem cells (Lin, 2002).

As we strive to unravel the pathways of cellular differentiation and proliferation within a tissue, we implicitly consider the stem cell as the governing body of these processes. Although stem cells are essentially the most important of all cells within a regenerating tissue, if we consider

their primitive, unspecialised characteristics, it is feasible that understanding the inner mechanics of a stem cell may be less important than elucidation of the nature of the cells which interact and regulate their behaviour i.e. the niche.

1.3) Cells of the small intestine and colon

1.3.1) Epithelial cells

Throughout the small intestine and colon, the internal luminal surface is lined by a simple, columnar epithelial mucosa with an underlying vascularised lamina propria, and muscularis mucosa. The nature and distribution of each cell type varies throughout each different functional region of the small intestine and colon, although each cell is believed to derive from a stem cell originating in the endoderm during embryogenesis (Maunoury et al., 1988). The epithelial mucosa has various structural specialisations, which considerably increase the luminal surface area to support the absorption and digestion of food.

In the small intestine and colon, frequent concave indentations known as "crypts" extend through the lamina propria down to the muscularis mucosae. In the small intestine, finger-like structures known as "villi" project into the lumen and expand the surface area. The fully-differentiated cells of the crypts and villi are situated towards the luminal surface, and are continually being shed into the lumen and replaced by a constant stream of proliferating progenitor cells. The columnar cells, termed "enterocytes" in the small intestine and "colonocytes" in the colon, are the most abundant epithelial cell type in the mucosa, responsible for secretion and absorption. Columnar cells are polarised cells with a basal nucleus and an apical brush border composed of microvilli, which increase the surface area further. The "goblet" cells, so-called due to their characteristic shape, are dispersed throughout the small intestinal and colonic epithelium and secrete mucus into the intestinal lumen to lubricate the mucosa and to trap and expel microorganisms. The gastrointestinal tract is the largest endocrine organ in the body, and hence the "endocrine", "neuroendocrine" or "enteroendocrine" cells are abundant throughout the epithelium, although in the small intestine are

more common to the crypts than the villi. To date, approximately 10 different endocrine cells have been described in the mammalian small intestine (Solcia et al., 1998), defined by their hormonal content, which is secreted from basally sited dense core or neurosecretory granules. The "Paneth" cells are almost exclusive to the crypt base of the small intestine and ascending colon. These cells maintain a sterile environment in the crypt *via* phagocytosis and the secretion of various antibacterial substances, including lysozyme, and antibacterial peptides called defensins or cryptdins from large apical secretory granules (Darmoul et al., 1997; Ouellette et al., 1994; Ouellette et al., 2000). Other less common cell lineages are also present, such as caveolated cells (Nabeyama and Leblond, 1974), and M- (membranous- or microfold-) cells which function in antigen transport, and are found in the small intestine within specialised lymphoid follicles, known as "Peyers patches" (reviewed in (Kerneis and Pringault, 1999)).

1.3.2) The lamina propria

The lamina propria is a layer of loose connective tissue, which lies immediately subjacent to the epithelial mucosa throughout the gastrointestinal tract. The lamina propria is composed of mesenchymal cells and their secreted basement membrane factors, and is highly vascular with lymphatic capillaries and nervous tissue (reviewed in (Kedinger et al., 1998; Powell et al., 1999a)). The lamina propria contains two intestinal myofibroblast populations: the interstitial cells of Cajal (ICC), and the intestinal subepithelial myofibroblasts (ISEMFs). The ICC are pacemaker cells located in an intramuscular space between the submucosa and muscularis propria, which regulate gastrointestinal smooth muscle motility and neurotransmission (Sanders, 1996). Other cell lineages present in the lamina propria include: fibroblasts and fibrocytes, vascular endothelial and smooth muscle cells, blood cell lineages such as granulocytes, mast cells, macrophages, T and B lymphocytes and plasma cells, verifying the role of the lamina propria as a key player in immune responses (Hunyady et al., 2000). The number

and distribution of cell types in the lamina propria, and their secretion of regulatory factors, varies in accordance to changing physiological conditions in the gastrointestinal tract (Hunyady et al., 2000).

1.3.2.1) The intestinal subepithelial myofibroblasts

ISEMFs display cytologic characteristics of both fibroblasts and smooth muscle cells, and are immunoreactive for α-smooth muscle actin (SMA), vimentin, and smooth muscle myosin antigens; though do not express desmin under normal circumstances (reviewed in (Powell et al., 1999a)). This antigenic phenotype permits their distinction from the vimentin-negative fibroblasts and the strongly desmin-positive smooth muscle cells. ISEMFs exist as a cellular syncytium in the subepithelial lamina propria, and are most prominently expressed around the lower two-thirds of the crypts. The cellular processes of ISEMFs have been shown to merge with the pericytes that surround the blood vessels (Joyce et al., 1987), and can encircle the capillaries that extend throughout the lamina propria in the small intestinal villi (Komuro and Hashimoto, 1990). ISEMFs proliferate and migrate upward along the crypt-villus axis until they reach the tip of the crypt or villi where they are then shed into the intestinal lumen, a process taking approximately 2 - 4 days in the mouse small intestine (Marsh and Trier, 1974b) and rabbit colon (Pascal et al., 1968). The pattern and timeframe of this myofibroblast migratory pattern is very similar to that of murine epithelial cells (Wright and Alison, 1984). ISEMFs are adjoined to epithelial cells *via* gap and adherens junctions, which permit epithelial: mesenchymal interaction (Powell et al., 1999b). ISEMFs have a broad range of functions including the regulation of epithelial cell homeostasis, mucosal protection and wound healing, contraction and motility of small intestinal villi and water and electrolyte transport in the colon (reviewed in (Powell et al., 1999b)).

The origin of the ISEMFs is unresolved, with reports of transdifferentiation between different mesenchymal cell types including resident tissue fibroblasts (Gabbiani, 1996) or smooth muscle cells (Ronnov-Jessen et

al., 1995), or a putative stem cell population in the lamina propria near the crypt base (Bockman and Sohal, 1998; Marsh and Trier, 1974a; Marsh and Trier, 1974b; Mayer-Proschel et al., 1997; Pascal et al., 1968; Sappino et al., 1989). However, more recent data from *in vitro* studies, indicates that cells in the BM can differentiate to produce myofibroblast-like cells (Ball et al., 2004; Emura et al., 2000; Kadner et al., 2002).

In normal, healthy tissue the ISEMF is present as a spindle-shaped, transiently differentiated cell. However, in inflamed tissue, ISEMFs become activated and assume a round, flattened morphology. Activated ISEMFs display an accelerated proliferation rate (McKaig et al., 2002), increased expression of cytokines, chemokines, growth factors and adhesion molecules, and have an enhanced secretion of soluble mediators of inflammation and extracellular matrix factors (reviewed in (Powell et al., 1999a; Powell et al., 1999b)). For example, activated ISEMFs stimulate epithelial cell hyperplasia in inflammatory bowel disease (IBD) by upregulating their secretion of the epithelial cell mitogen, keratinocyte growth factor (KGF) (Bajaj-Elliott et al., 1997). Furthermore, overactivation and persistence of activated ISEMFs causes tissue fibrosis and scarring in Crohn's disease (Aigner et al., 1997; Graham, 1995; Isaji et al., 1994; Kinzler and Vogelstein, 1998; Martin et al., 1996).

1.4) Intestinal epithelial stem cells

The adult epithelial cells of the intestinal mucosa are constantly under high regenerative pressure due to the rapid rate of cell turnover in this tissue. For example, the lining of the gastrointestinal tract is replaced every 2 – 3 days in rodents and other mammals (Wright and Alison, 1984). To regulate homeostasis, a vital balance between cell apoptosis, senescence and the proliferation and differentiation of new cells must be maintained. This role is attributed to the intestinal stem cell although, despite its significance as the most important regulatory element of intestinal function, limited evidence exists to definitively substantiate the location, quantity, regulatory pathways or function of this elusive cell.

1.4.1) Intestinal epithelial stem cell location

Since the polarised orientation of the differentiated cells in the intestinal crypts and villi is well characterised, it is presumed that a pool of progenitor cells exist at the origin of cellular migration and are responsible for this continuous cellular flux. The epithelial cells of the small intestine originate in the lower regions of the crypt, and undergo proliferation and differentiation as they travel upwards to be shed into the intestinal lumen, with the exception of the Paneth cells, which migrate downward to their location in the crypt base. It is therefore generally accepted that the epithelial stem cell compartment is in the crypt base of the colon, and at approximately cell position 4 - 5 in the small intestinal crypts, just superior to the Paneth cells (Figure 1.1; (Potten et al., 1997)).

1.4.2) Intestinal epithelial stem cell niche

As with stem cells of most adult tissues, it is thought that the intestinal epithelial stem cells are located and maintained within a mesenchymal niche, situated near the base of the intestinal crypts. However, as the

stem cells remain unidentified, it is not possible to localise the niche that underlies them and thus, our knowledge of the intestinal niche is fundamentally based upon circumstantial evidence and speculation. Ablation studies of specific epithelial cell lineages have failed to identify which cells comprise and are targeted by the intestinal niche. For example, targeted ablation of Paneth cells (Garabedian et al., 1997), and three different endocrine cell lineages in the mouse small intestine (Rindi et al., 1999), had no effect on the rate of epithelial cell proliferation. It is more conceivable that epithelial stem cells are regulated by non-epithelial components i.e. the mesenchymal cells and ECM factors in the subjacent lamina propria, which fits with the classical view of the composition of the stem cell niche (Ohlstein et al., 2004; Spradling et al., 2001), and is further supported by evidence of interactions between the ISEMFs and epithelial cells of the mucosa. For example, ISEMFs play vital roles in epithelial cell restitution, remodelling, fibrosis and immunological and inflammatory responses (reviewed in (Powell et al., 1999b), in their secretion of specific growth factors and inflammatory cytokines (Dignass and Sturm, 2001; Dignass et al., 1994; Podolsky, 1997; Polk, 1998; Riegler et al., 1996). This epithelial:mesenchymal interaction highlights the ISEMF as a candidate component of the intestinal epithelial stem cell niche, although it is not clear if mesenchymal signals act directly upon the stem cells or the transiently-differentiated daughter cells.

1.4.3) Intestinal stem cell number/ clonal origins of intestinal crypts

1.4.3.1) Mouse

The number of stem cells within the gastrointestinal niche is a subject of ongoing debate. Investigations into the clonal origins of intestinal crypts have attempted to deduce whether they are monoclonal populations derived from a single stem cell, or whether multiple stem cells proliferate to produce polyclonal crypts. The "Unitarian hypothesis" states that a single intestinal stem cell can clonally expand to produce the entire adult cell repertoire in the intestinal crypts (Cheng and Leblond, 1974). This is supported by evidence that a single surviving stem cell can recreate entire monoclonal crypts following irradiation damage (Ponder et al., 1985), although these studies of damaged epithelium do not confirm that intestinal crypts are monoclonal under normal circumstances.

Mouse aggregation chimeras, wherein one parental strain bears a specific marker for the tissue of interest, have been used to investigate the Unitarian hypothesis. The binding capacity of the *Dolichos biflorus* agglutinin (DBA) lectin to the cells of the intestinal epithelium can be abolished by spontaneous mutation of the *Dlb-1* locus on chromosome 1, or by treatment with the chemical mutagen, ethylnitrosourea (ENU). The SWR mouse has a carbohydrate polymorphism of *Dlb-1*, and consequently, DBA binds to sites on the C57Bl/6J-derived, but not SWR-derived cells in heterozygous C57Bl/6J(B6)↔SWR mouse embryo aggregation chimeras. Intestinal crypts in neonatal B6↔SWR mice were polyclonal for the first two weeks after birth, suggesting that multiple stem cells exist during development (Schmidt et al., 1988). However, all crypts eventually become monoclonal, possibly due to the positive selection of a single dominant clone or by the segregation of lineages due to the division of crypts by “crypt fission”, which occurs frequently during this

developmental period (section 1.5.1;(Bjerknes and Cheng, 1999; Park et al., 1995)). The epithelial cells in the crypts remain monoclonal in the adult mouse intestine, confirming the existence of a single, sustainable stem cell (Bjerknes and Cheng, 1999; Ponder et al., 1985; Winton and Ponder, 1990). Similarly, ENU treatment in B6↔SWR mice results in loss of DBA binding in mutated cells, and revealed that small intestinal crypts are initially partially, and then entirely negative for DBA staining (Winton et al., 1988). It is proposed that ENU causes mutation of the *Dlb-1* locus in a stem cell, which then expands stochastically to produce a clone of cells that cannot bind DBA and remain unstained (Winton et al., 1988). If this is the case then a single stem cell can give rise to all the epithelial lineages within the small intestinal crypts.

In female mice with a heterozygous polymorphism of the X-linked gene, glucose-6-phosphate dehydrogenase (*G6PD*), individual X chromosomes are distinguished by their natural "mosaic" pattern of *G6PD* immunohistological staining. Use of this model to investigate clonality in the intestinal crypts, excludes the possibility that crypts derived from distinct strains in chimeric mice segregate differentially during organogenesis. The monophenotypic origin of murine intestinal crypts was confirmed following histochemical analyses in these mice, although the small intestinal villi showed a polyclonal derivation, and are presumably formed by the upwards migration of epithelial cells from multiple crypts (Thomas et al., 1988). This is concordant with observations that crypts, although smaller than villi, are 7-fold more numerous in the mouse duodenum, and 4-fold in the ileum (Wright and Alison, 1984).

In both chimeric mice and the *G6PD* mouse model, the time taken for the decrease in partially-mutated crypts and emergence of entirely negative crypts to reach a plateau was approximately 4 weeks in the small intestine and 12 weeks in the large intestine, which was initially thought to be due to tissue differences in cell cycle duration. However, an alternative explanation can be found in the “stem cell zone” hypothesis,

which was conceived following a series of microscopic morphometry and autoradiography-labelling studies of the mouse small intestine (Bjerknes and Cheng, 1981a; Bjerknes and Cheng, 1981b; Bjerknes and Cheng, 1981c; Bjerknes and Cheng, 1981d; Bjerknes and Cheng, 1981e). In this hypothesis, multiple stem cells occupy the crypt base at cell positions 1 – 4, and can undergo proliferation but do not differentiate until they have migrated to cell position 5. Paneth cells also appear initially at position 5, although these cells migrate downward to the crypt base (Bjerknes and Cheng, 1981a). Based upon this hypothesis, it was suggested that larger numbers of stem cells are present in the colon than in the small intestine, causing the difference in time taken for the mutant stem cells to expand stochastically and create monoclonal crypts (Williams et al., 1992).

Alternatively, the temporal differences in the appearance of monophenotypical crypts in specific regions of the gastrointestinal tract may be due to differential rates of crypt fission at the time of mutagen administration (Park et al., 1995). It was suggested that stem cell number fluctuates throughout the crypt cycle until a threshold number of cells is reached, thereby signalling for crypt fission to occur (Loeffler et al., 1997). Other reports state that between 4 - 6 stem cells located at cell position 4 - 5 from the base of the crypt, just superior to the Paneth cells, comprise the small intestinal stem cell population (Cai et al., 1997; Potten, 1998; Potten et al., 1997), and others claim that up to 16 or more stem cells can exist in a single intestinal crypt (Roberts et al., 1995).

1.4.3.2) Human

In humans, the intestinal crypts appear to be monoclonal and the small intestinal villi are polyclonal, analogous to the situation in the mouse. The single cell-cloned human colorectal carcinoma cell line, HRA19, was derived from a primary adenocarcinoma of the rectum. A colony of single morphology was cloned and grown as a monolayer *in vitro*. When engrafted subcutaneously into nude mice, the clones produce tumours that were histologically identical to the founding tumour and contain each

major epithelial cell type found *in vivo* (Kirkland, 1988). However, it is important to note that the results of this study of neoplastic cells cannot be directly applied to the normal human gastrointestinal epithelia.

Perhaps the best evidence for the clonal origin of human intestinal crypts comes from a study of the colon of a rare XO/XY patient who had received a prophylactic colectomy for familial adenomatous polyposis (FAP). Non-isotopic *in situ* hybridisation (NISH) using Y chromosome-specific probes showed that normal intestinal crypts were composed almost entirely of either Y chromosome-positive or Y chromosome-negative cells, with approximately 20% of crypts being XO. Immunostaining for neuroendocrine cell-specific markers was combined with NISH detection of the Y chromosome to show that these cells shared the same genotype as other crypt cells. The small intestinal villi epithelia were a mixture of XO and XY cells, in keeping with the notion that villi derive from stem cells of more than one crypt. Of the 12,614 crypts examined, only 4 crypts were composed of XO and XY cells, which was explained by non-disjunction with loss of the Y chromosome in a crypt stem cell. These observations agree with previous findings in chimeric mice that intestinal crypts are monoclonal and derive from a single multipotential stem cell.

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 that is derived from either a single clone or from the coalescence of multiple clones of the same lineage (Garcia et al., 2000; Schmidt et al., 1985). Clonality must be determined at the patch edge, as it is not possible to decipher whether 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 9 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 2,260 crypts located at the periphery of a patch, indicating that colonic crypts are indeed monoclonally-derived (Novelli et al., 2003). The Unitarian hypothesis, that all the differentiated epithelial lineages within the gastrointestinal tract share a common cell of origin, appears to apply to both mice and humans.

1.5) Methods of identifying intestinal stem cells

The maintenance of homeostasis of the intestinal epithelium is an intricate and complex interplay of multiple regulatory mechanisms. It is therefore of great interest to uncover the molecular and cellular pathways that are required for normal intestinal function, starting, of course, with the ongoing quest to identify the intestinal epithelial stem cell and its niche.

1.5.1) Crypt fission and the crypt cycle

The intestinal crypts divide and replicate by a branching process known as "crypt fission". In this process, the intestinal crypts undergo basal bifurcation and budding, leading to longitudinal division and the formation of identical daughter crypts. Elucidation of the process of crypt multiplication, led to the notion that the intestinal crypts have a "crypt cycle" and finite lifespan, which was subsequently shown to be approximately 2 years in the mouse small intestine (Loeffler et al., 1997; Totafurno et al., 1987). As crypt fission begins at the bottom of the crypt i.e. the location of the putative stem cells, it is possible that the stem cells initiate and regulate the formation of new crypts. Indeed, evidence is emerging that crypt fission may occur in the intestine when a maximal crypt size is reached (Park et al., 1997), possibly when a threshold number of stem cells becomes exceeded (Loeffler and Grossmann, 1991).

A stochastic state dependent model of stem cell growth, proposes that there are 3 possible outcomes when stem cells divide, described as; "r", wherein a stem cell divides asymmetrically to produce one stem cell that is retained within the niche and one daughter cell that leaves the niche and becomes committed to differentiation; "p", wherein 2 stem cells are produced following symmetrical stem cell division and; "q", where stem cells divide symmetrically to produce two daughter cells that leave the

niche and become transit amplifying cells (Figure 1.2; (Loeffler et al., 1993)).

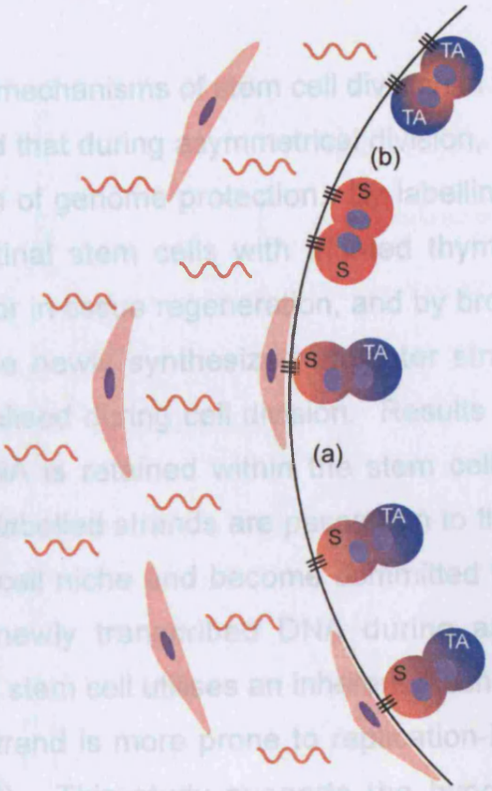

Figure 1.2) Possible outcomes of stem cell division.

A stem cell (s) can divide asymmetrically to produce one stem cell that is retained within the niche and one transit amplifying (TA) cell that leaves the niche and becomes committed to differentiation (a). Alternatively, a stem cell can undergo symmetrical division to produce two identical stem cells, or two TA daughter cells (b). Diagram courtesy of MJ Lovell.

1.5.2) DNA-labelling studies

A recent study of the mechanisms of stem cell division in the mouse small intestine has revealed that during asymmetrical division, stem cells retain an innate mechanism of genome protection. By labelling DNA template strands of the intestinal stem cells with tritiated thymidine ([3H]TdR) during development or in tissue regeneration, and by bromodeoxyuridine (BrdUrd)-labelling the newly synthesized daughter strands, both DNA strands can be visualised during cell division. Results showed that the original template DNA is retained within the stem cell, and the newly synthesised BrdUrd-labelled strands are passed-on to the daughter cells that leave the stem cell niche and become committed to differentiation. By discarding the newly transcribed DNA during asymmetrical cell division the intestinal stem cell utilises an inherent mechanism of genome protection, as this strand is more prone to replication-induced mutation (Potten et al., 2002). This study supports the hypothesis of Cairns (Cairns, 1975), which suggests that selective retention of the template DNA strand during stem cell division provides a means of protection against DNA replication errors (Figure 1.3).

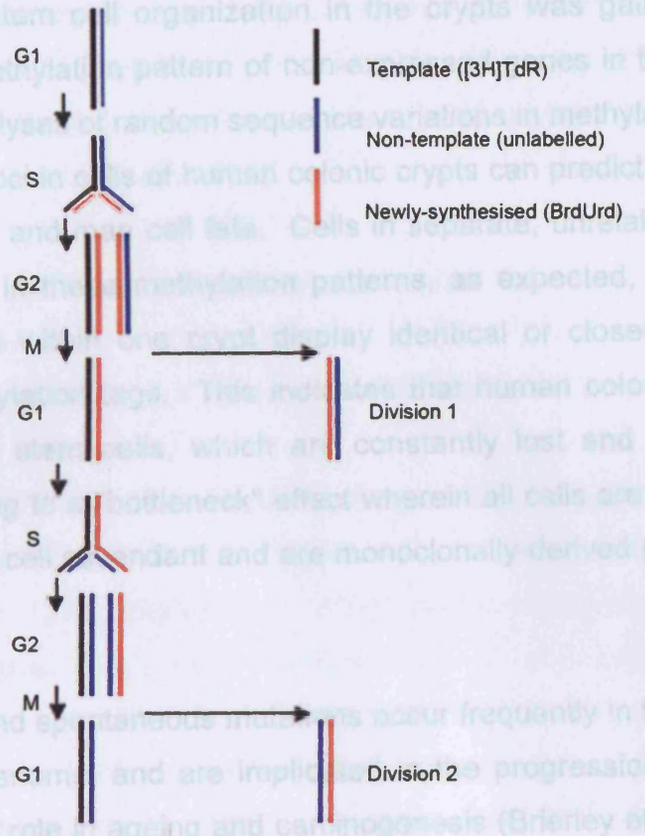

Figure 1.3) The segregation of template and newly-synthesised DNA strands in a chromosome during cell division, according to the Cairn's hypothesis.

Template DNA strands are selectively retained by the stem cell during cell division, whereas the newly-synthesised DNA strands are segregated to the daughter cell destined to enter the dividing transit compartment and be shed from the tissue after a few days, thus removing any replication-induced errors. Label introduced into the newly-synthesised DNA takes two divisions to be removed from the stem cells, whereas label in the template strand would persist in the stem cell line. (Adapted from (Potten et al., 2002)).

1.5.3) DNA mutation studies

An insight into stem cell organization in the crypts was gained from studies of the methylation pattern of non-expressed genes in the colon. Phylogenetic analyses of random sequence variations in methylation tags of three neutral loci in cells of human colonic crypts can predict stem cell division histories and map cell fate. Cells in separate, unrelated crypts show variations in these methylation patterns, as expected, and cells closely opposed within one crypt display identical or closely related sequential methylation tags. This indicates that human colonic crypts contain multiple stem cells, which are constantly lost and replaced, eventually leading to a "bottleneck" effect wherein all cells are related to the closest stem cell ascendant and are monoclonally-derived (Yatabe et al., 2001).

Both inherited and spontaneous mutations occur frequently in the human mitochondrial genome, and are implicated in the progression of many diseases, with a role in ageing and carcinogenesis (Brierley et al., 1998; Coller et al., 2001; Michikawa et al., 1999). Mathematical models indicate that the high frequency of mitochondrial DNA (mtDNA) mutations, and their accumulation within individual cells is a result of clonal expansion by genetic drift (Chinnery and Samuels, 1999; Coller et al., 2001), and can produce a defect in oxidative phosphorylation (Sciacco et al., 1994). Cells demonstrating >50% cytochrome-c oxidase enzyme function and normal succinate dehydrogenase (SDH) activity generally have increased numbers of mtDNA mutations (Johnson et al., 1993). The biochemical function of samples of both normal and cancerous human colonic mucosa was deduced by sequencing their mtDNA, and by histological analysis of cytochrome-c oxidase and SDH function. The results were inconsistent, as crypts were composed either entirely, or partially, of cells with mtDNA mutations, suggesting that a single stem cell exists in the wholly mutated crypts, and that multiple stem cells co-exist in the mixed crypts (Taylor et al., 2003). Mitochondrial DNA mutations accumulate in stem cells in the colonic crypts in an age-dependent fashion and can produce a

biochemical deficiency dependent on the site and severity of the mutation. This study has implications for the role of mtDNA mutations in the onset of cancer, and presents a link between mutations in mtDNA and the resultant aberrant oxidative phosphorylation in cancer cells, as accumulating mutations occur within crypt stem cells, which then undergo clonal expansion (Taylor et al., 2003).

1.5.4) Molecular markers of intestinal stem cells

1.5.4.1) Musashi and Hairy and Enhancer-of-split proteins

Musashi-1 (Msi-1) is the mammalian homologue of a Drosophila protein required for asymmetric division of sensory neural precursor cells (Nakamura et al., 1994; Okabe et al., 2001), and is highly expressed in mammalian neural stem cells (Sakakibara et al., 1996). The transcriptional repressor Hairy and Enhancer-of-split (Hes)-1 is essential for neural stem cell self-renewal and suppression of neural stem cell differentiation (Akazawa et al., 1992; Nakamura et al., 2000; Sasai et al., 1992). Msi-1 positively regulates transcription of Hes-1, suggesting a close interaction between the 2 proteins (Imai et al., 2001). This is supported by their co-expression in cells just superior to the Paneth cells in the small intestine, the postulated stem cell zone. Hes-1 expression in the mouse small intestine is more widespread than Msi-1 expression, as Hes-1 is also expressed, albeit in reduced levels, in epithelial cells migrating toward the villus tip. It is suggested that co-localisation of Msi-1 and Hes-1 in cells just superior to the 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 (Kayahara et al., 2003). Musashi-1 mRNA and protein expression has also been confirmed in the putative stem cells in neonatal and adult intestinal crypts in mice (Potten et al., 2003), and has recently been demonstrated in the human colon in epithelial cells located between position 1-10 in the crypts (Nishimura et

al., 2003). These studies implicate Musashi-1 as a possible gastrointestinal epithelial stem cell marker.

1.5.4.2) Repression of E-cadherin

E-cadherin is a cell adhesion molecule with a well-established role in tumour suppression. Downregulation of E-cadherin expression is implicated in a number of pathological conditions in the gut, which involve increased cell proliferation, loss of cell polarity and/or differentiation, including IBD and cancer (Del Buono and Pignatelli, 1999; Gagliardi et al., 1995; Gassler et al., 2001; Hanby et al., 1996). E-cadherin is expressed in high levels in the intestinal epithelium, and has been shown to regulate intestinal cell polarity and differentiation both *in vitro* (Schreider et al., 2002) and *in vivo*, *via* the β-catenin/Tcf/LEF signalling pathway (Gottardi et al., 2001; Stockinger et al., 2001), which is vital for normal intestinal epithelial cell function (section 1.6.1). A recent publication claims that differential E-cadherin expression in the epithelial cells along the human small intestine crypt-villus axis provides an insight into the nature of the hierarchical polar distribution of these cells, and can be used to locate the intestinal epithelial stem cells. E-cadherin is absent from the cells in the base of the human small intestinal crypts, at approximately cell position 5 – 7 i.e., the stem cell zone. Forced expression of E-cadherin in those cells caused a decrease in proliferation, and promoted cell junction formation thereby inhibiting cell migration. It is therefore proposed that E-cadherin represents a marker of intestinal epithelial stem cells, and plays an important role in regulating stem cell behaviour. However, the inability of E-cadherin to induce terminal differentiation of enterocytes indicates that it acts in conjunction with other proteins (Escaffit et al., 2005).

1.6) Pathways of cellular differentiation in the intestine

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 function in both the normal and neoplastic gastrointestinal mucosa. The ongoing elucidation of these molecular pathways is key to providing an insight into the location and the behaviour of the intestinal epithelial stem cell.

1.6.1) The Wnt/β-catenin signalling pathway

The central player in the canonical Wnt signalling pathway is the cytoplasmic protein beta (β)-catenin, whose stability is regulated by the APC tumour suppressor complex. When Wnt receptors are not engaged, APC forms a subcellular, trimeric complex with axin and glycogen synthase kinase-3β (GSK3β), which triggers the phosphorylation, ubiquitination and proteosomal degradation of β-catenin. Wnt stimulation activates the cytoplasmic phosphoprotein "dishevelled" through its receptor "frizzled", causing inhibition of GSK3β and a resultant accumulation of cytosolic β-catenin (Kinzler and Vogelstein, 1996). Beta-catenin then translocates into the nucleus and interacts with members of the T cell factor/lymphoid-enhancing factor (Tcf/LEF) family of DNA-binding proteins, transiently converting them from transcriptional repressors to activators, and signalling the activation of downstream target genes that increase cellular proliferation. When the Wnt signal is removed, APC extracts β-catenin from the nucleus and the transcriptional repressor function of Tcf is restored (reviewed in (Bienz, 2002; Sancho et al., 2004)).

The Wnt/β-catenin pathway plays a role in malignant transformation, as 85% of human sporadic colorectal tumours are reported to have an APC

mutation (Kinzler and Vogelstein, 1996). This APC mutation renders the GSK3β/Axin/APC complex incapable of destabilising β-catenin, leading to an accumulation of nuclear β-catenin/Tcf/LEF complexes, and an increase in target gene transcription and cell proliferation which can cause tumour formation (Figure 1.4; (Mei et al., 1999; Wielenga et al., 1999)).

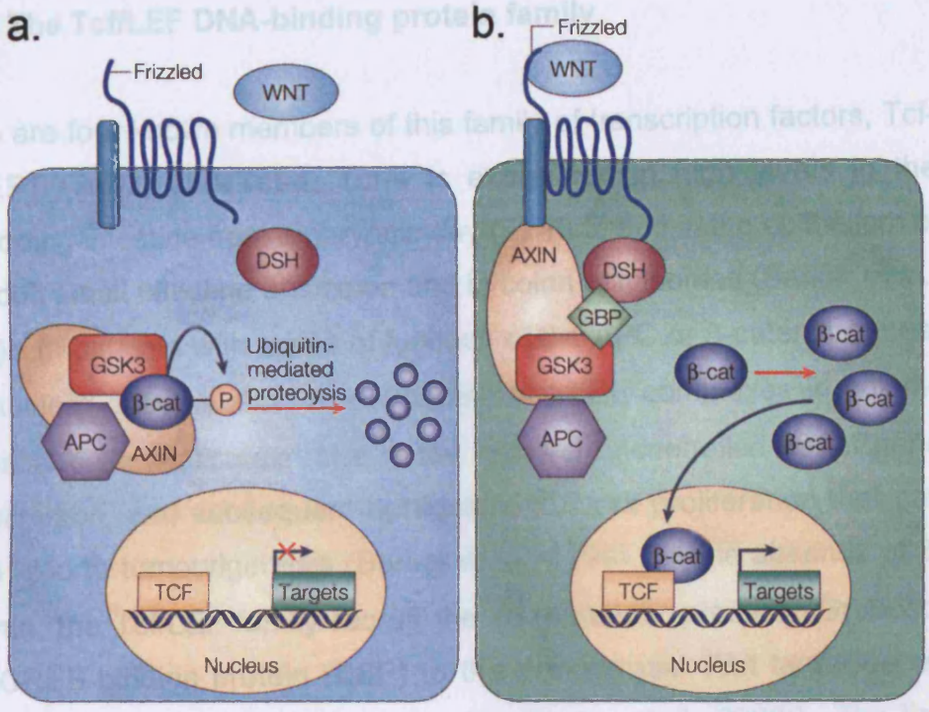

Figure 1.4) The canonical Wnt/β-catenin signalling pathway.

In the absence of active Wnt signalling (**a.**), APC forms a complex with axin and GSK-3, which triggers the degradation of β-catenin, and maintains prospective target genes in a repressed state. Wnt signalling (**b.**), activates the cytoplasmic phosphoprotein DSH, through its receptor "Frizzled", to reduce β-catenin degradation. As β-catenin accumulates, it enters the nucleus and interacts with members of the T-cell factor (TCF) and lymphoid-enhancing factor (LEF) family of transcription factors to activate the transcription of target genes. APC, adenomatous polyposis coli; β-cat, β-catenin; DSH, dishevelled; GSK-3, glycogen synthase kinase-3β; P, phosphorylation; TCF, T-cell factor. (Adapted from (Moon et al., 2004)).

1.6.2) 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 embryonic day (E) 13.5, and in the epithelium of the adult small intestine and colon and in colon carcinomas (Barker et al., 1999). In patients with a loss of function of the APC or β-catenin genes, the nuclear accumulation of β-catenin/Tcf-4/LEF complexes in colonic epithelial cells implicates Tcf-4 in the ensuing uncontrolled target gene transcription, and subsequent upregulation of cell proliferation that can often lead to tumourigenesis (Barker et al., 1999). In the absence of β-catenin, the Tcf/LEF family recruit the co-repressor proteins "Groucho" and CREB-binding protein (CBP) to the downstream Wnt target genes and inhibit their transcription (reviewed in (Barker et al., 2000)). 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 postulated that Tcf-4 is vital for the establishment of the intestinal stem cell population, and is activated by a Wnt signal from the underlying stroma (Korinek et al., 1998), further evidence that mesenchymal cells in the lamina propria comprise the intestinal stem cell niche.

TCF target genes include; *c-myc, tcf-1, cyclin D1, c-Jun, Fra-1*, urokinase-type plasminogen activator receptor *(uPAR), fibronectin, CD44,* and the matrix metalloproteinase *matrilysin*, many of which have carcinogenic potential in the gastrointestinal tract (reviewed in (Bienz and Clevers, 2000; Polakis, 1999)). Two important Tcf-4 target genes are the tyrosine kinase guidance receptors *EphB2* and *EphB3*, which are expressed along a gradient with the highest levels found in the crypt base, whereas levels of their ligand ephrin-B1 increase in a counter-gradient along the crypt-villus axis. In mice with targeted deletion of *EphB2* and *EphB3* genes, differentiated epithelial cells lose their

polarised organisation resulting in a random distribution of mucosal cells (Batlle et al., 2002).

1.6.3) Cdx-1 and Cdx-2 homeobox genes

The mammalian homeobox proteins Cdx-1 and Cdx-2 also 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 (Subramanian et al., 1998), and both Cdx-1 and Cdx-2 mRNAs show restricted expression in the epithelial mucosa of the human small intestine and colon (Mallo et al., 1997; Mizoshita et al., 2001). 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 development of the epithelial stem cell niche (Lickert et al., 2000). Expression of Cdx-1 is reduced in proliferating epithelial cell nuclei in colonic crypts concurrent with their progression to adenomas and adenocarcinomas (Lickert et al., 2000) although, as no colonic tumours develop in the Cdx-1 null mouse, this molecule does not appear to have direct tumour suppressing properties (Subramanian et al., 1998). 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 (Ee et al., 1995). 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 function.

1.6.4) 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 (reviewed in (Kaestner et al., 2000)). 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 sulfate proteoglycans (HSPGs), causing overactivation of the Wnt/β-catenin pathway and an increase in target cell proliferation, demonstrating an indirect regulation of the Wnt/β-catenin pathway by Fox1-mediated HSPG production (Kaestner et al., 1997).

1.6.5) The TGF-β/Smad signalling pathway

The transforming growth factor TGF-β family of growth factors are known inhibitors of gastrointestinal epithelial cell proliferation, and are predominantly expressed in the differentiated compartments of the gut (Winesett et al., 1996). Under normal circumstances, TGF-β forms a multimeric complex with two serine-threonine kinase surface receptor molecules, TGF-β type I (TGFβRI) and type II (TGFβRII). The intracellular messengers of the TGF-β signalling pathways are the cytoplasmic Smad proteins, and Smad2 and Smad3 are phosphorylated upon activation of the TGF-β receptors, and form a heteromeric complex with Smad4. This complex then translocates to the nucleus, where it interacts with transcriptional coactivators and corepressors to regulate TGF-β target gene transcription (Fiocchi, 2001; Miyazono, 2000).

Disruption of TGF-β/Smad signalling causes intestinal epithelial cell hyperproliferation, and Smad2, Smad4 and TGFβRII are frequently inactivated in human cancers confirming their function as tumour suppressor genes (Grady et al., 1999; Markowitz et al., 1995; Massague, 1998). Lack of TGF-β signalling in the intestine appears to contribute to the progression of early pre-existing lesions (reviewed in (Sancho et al., 2004)). For example, mice with targeted heterozygous 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 rapidly progress to form adenocarcinomas with an increased malignant nature than those formed in mice with a heterozygous mutation of the APC allele only (Takaku et al., 1998). This 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 (Takaku et al., 1998).

1.6.6) The Notch signalling pathway

Notch encodes a large, single transmembrane receptor, of which there are four mammalian isoforms (Notch 1 – 4) and five corresponding ligands (Delta-like 1, 3, and 4; Jagged 1 and 2) (reviewed in (Sancho et al., 2004)). The Notch-signalling pathway regulates gastrointestinal epithelial cell fate and the 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 (Cheng and Leblond, 1974). Increased levels of Notch protein negatively regulate the transcription of the *Math1* gene, a basic loop-helix transcription factor, *via* an upregulation of the *Hes1* transcriptional repressor. Mice with a 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 (Yang et al., 2001). Conversely, a 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 (van Den Brink et al., 2001). *Hes1* knockout mice display an elevated Math1 expression with a concurrent increase in the numbers of goblet, Paneth and enteroendocrine cells and a reduced enterocyte population (Jensen et al., 2000). These results suggest that the absorptive versus secretory cell fate decision is established through the Notch-Hes1

pathways, although aberrant Notch signalling in intestinal tumourigenesis has not been described to date (reviewed in (Sancho et al., 2004)).

1.7) Cells of the epidermis

The mammalian epidermis is the outer covering of the skin, composed of a stratified squamous, keratinised epithelium and its associated appendages, the hair follicles, sebaceous glands and sweat glands. The epidermis is subdivided into a basal layer, spinous layer, granular layer and the peripheral stratum corneum, which is in contact with the outer environment. The keratinocytes are the principal epithelial cell lineage in the epidermis, so-called because of the family of filamentous proteins, the keratins, which comprise its distinctive cytoskeleton. Other cells in the epidermis are the Langerhans cells, which provide immunological protection, the melanocytes that function in their absorption of ultraviolet light, and the Merkel cells, which act as sensory mechanoreceptors (reviewed in (Leigh et al., 1994)). The basal epidermis rests on a basement membrane, which overlies the dermis, a dense fibroelastic connective tissue composed of collagen and elastic fibres that encloses the vasculature and the nervous system of the skin.

1.7.1) Keratinocytes

The basal layer of the epidermis contains the epidermal stem cells and their transit amplifying cell progeny, which leave the niche and travel to the surface of the skin, giving rise to the entire differentiated keratinocyte repertoire during this hierarchical migration. Basal cells are distinguished by an intracellular cytoskeleton composed of a relatively dispersed, but extensive, network of keratin filaments. The spinous layer, or stratum spinosum, is 4 - 8 cells wide in humans. The keratinocytes in this layer contain large, dense bundles of keratin intermediate filaments, which extend to the cell periphery and form desmosomal cell junctions between adjacent cells, thereby imparting mechanical strength to the tissue. The spinous cells gradually become more flattened as they migrate upward, and express lamellar granules that secrete specific lipids and enzymes,

which contribute to the barrier function of the epidermis by filling the intercellular spaces of the stratum corneum. The granular layer, or stratum granulosum, is generally comprised of 2 – 3 cell layers in humans, although this is markedly increased in the "ridged" skin of the palms and soles. The keratinocytes in this layer are post-mitotic and are distinguished by the presence of keratohyalin granules. Granular cells also synthesise and crosslink a number of structural proteins to form the cornified cell envelope of the terminally differentiated, non-viable stratum corneum. The human stratum corneum consists of 15 – 20 layers of flat, hexagonal-shaped anucleate cells, or squames. When keratinocytes enter the stratum corneum, the process of "keratinisation" is complete, and cells are eventually sloughed from the epidermal surface. The time taken for a keratinocyte to differentiate from stem cell to squame is approximately 8 – 9.5 days in mice (Potten et al., 1987), and between 28 – 60 days in humans (Hunter et al., 1995).

1.7.2) Non-keratinocyte lineages

The melanocytes, Langerhans and Merkel cells comprise the non-keratinocyte component of the mammalian epidermis, accounting for less than 20% of all epidermal cells. These cells assume a non-random distribution amongst the keratinocytes throughout the epidermis; melanocytes and Merkel cells are typically present in the basal layer, whilst the Langerhans cells are found suprabasally in the spinous and granular layers. The melanocytes are dendritic-like cells, which are easily distinguished from keratinocytes by their lack of keratin intermediate filament bundles and by the presence of small, membrane-bound melanin pigment-containing melanosomes. Melanocytes are present in a constant ratio of 1 melanocyte to 36 keratinocytes, the "epidermal melanin unit" (Fitzpatrick and Breathnach, 1963; Jimbow et al., 1976), and function in their transfer of melanin to keratinocytes *via* dendritic processes. The Langerhans cells also have a dendritic phenotype and are specialised in the uptake, processing and presentation of specific antigens. These cells are distinguished by their lack of keratin

intermediate filament bundles and the presence of "Birbeck granules", which form by invagination of the plasma membrane to enclose bound antigen (Takahashi and Hashimoto, 1985). The Merkel cells are the hormone-secreting cells of the epidermis, which function as slow-adapting mechanoreceptors (Halata et al., 2003). Merkel cells contain small, membrane-bound dense core granules and can synthesise keratin intermediate filaments, although unlike the bundles of intermediate filaments in keratinocytes, these are loosely distributed within the cytoplasm. Merkel cells are the least common non-keratinocyte lineage in the epidermis, although are expressed in higher numbers in the palms, buccal mucosa, lips and soles i.e., regions of high touch sensitivity (Lacour et al., 1991).

1.7.3) The pilosebaceous hair follicle

1.7.3.1) The hair follicle

Hair follicles are the major appendage in mammalian skin, and are comprised of an epithelial core surrounded by a mesenchymal sheath. The epithelial core is composed of several specialised layers including, the outer root sheath (ORS), which is continuous with the overlying interfollicular epidermis (IFE), and the inner root sheath (IRS) that includes, from centre to periphery, the cuticle and Henle and Huxley's layers. The lower portion of the hair follicle protrudes into the dermis and is dilated into a bulb of epithelium enclosing the dermal papilla, a specialised pocket of mesenchymal cells. The hair shaft is enclosed within the IRS, and develops from progenitor cells located in the matrix region surrounding the dermal papilla (reviewed in (Goldsmith, 1991)).

Hair follicles undergo a constant cycle of alternating growth (anagen), regression (catagen) and rest (telogen). In the mouse, anagen lasts approximately 3 weeks, and the first 3 cycles are relatively synchronous. In humans, anagen can last for several years in hair follicles in the scalp, whereas the growth period for the eyelashes and eyebrows is around 1 –

4 months, although the hair cycle is not synchronous in the skin of humans as each follicle maintains an independent rhythm of growth and rest (reviewed in (Alonso and Fuchs, 2003)).

1.7.3.2) The sebaceous glands

The sebaceous glands are attached to the upper permanent portion of the hair follicle where they are unaffected by the hair cycle and epidermal wounding. Sebaceous glands are associated with hair follicles all over the body, with the exception of the palms and soles, which are devoid of hair. Glands can be unilobular or multilobular, and vary greatly in size independent of their location, although the greatest density of glands are on the face and scalp. As the sebaceous cells, or sebocytes, migrate from the basal layer toward the centre of the gland, they accumulate a lipid-rich fluid, or sebum, that is eventually secreted onto the epidermal surface *via* the sebaceous duct. As sebocytes approach the sebaceous duct they become distended due to their high sebum content, and their nuclei and other intracellular structures disappear. The cells eventually rupture and release their contents into the duct by holocrine secretion (reviewed in (Thiboutot, 2004)).

1.8) Epidermal stem cells

The cells of the adult epidermis and its associated appendages undergo continual renewal and remodelling throughout life, and therefore a strict balance between cell proliferation and differentiation must be maintained by the endogenous stem cells. Most adult stem cells divide infrequently and thus have a long cell cycle time e.g., stem cells of the skin and gut have a cell cycle time approximately 2-fold slower than their transit amplifying cell progeny, although cell cycle time is dramatically reduced following damage or disease e.g., mouse stem cell cycle times can be reduced from 200 hours to 12 hours following epidermal wounding (reviewed in (Potten and Booth, 2002)). The stochastic state-dependent model of intestinal stem cell growth proposed by Leoffler et al., (section 1.5.1; (Loeffler et al., 1993)), can also be applied to the epidermal stem cells, wherein three possible outcomes are possible following stem cell division. Stem cells can undergo symmetrical division to produce two identical stem cells that are retained within the stem cell niche, or can divide asymmetrically to produce one identical stem cell and one daughter cell that leaves the niche and becomes committed to differentiation, or can undergo a third type of division to produce two daughter transit amplifying cells that leave the niche and differentiate to form specific epidermal lineages (reviewed in (Brouard and Barrandon, 2003)).

A well-established model of DNA labelling can be used to localise the stem cells within a tissue, taking advantage of their long cell cycle. It is possible to label all the dividing cells in the developing or regenerating epidermis by a repeated or continuous supply of either BrdUrd or [3H]TdR, followed by a long chase period. The transit amplifying cells divide to dilute and eventually lose their label during the chase period, and only the cells that have a very slow cycle retain the label i.e., the stem cells.

The epidermal stem cells reside in the basal layer of the epidermis, and are believed to inhabit stem cell niches in two separate locations, the "bulge" region of the hair follicles in both mice and humans, and the epidermal proliferative units (EPUs) in the rodent IFE, and in the basal epidermis at the top of the rete ridges in human IFE (Figure 1.5).

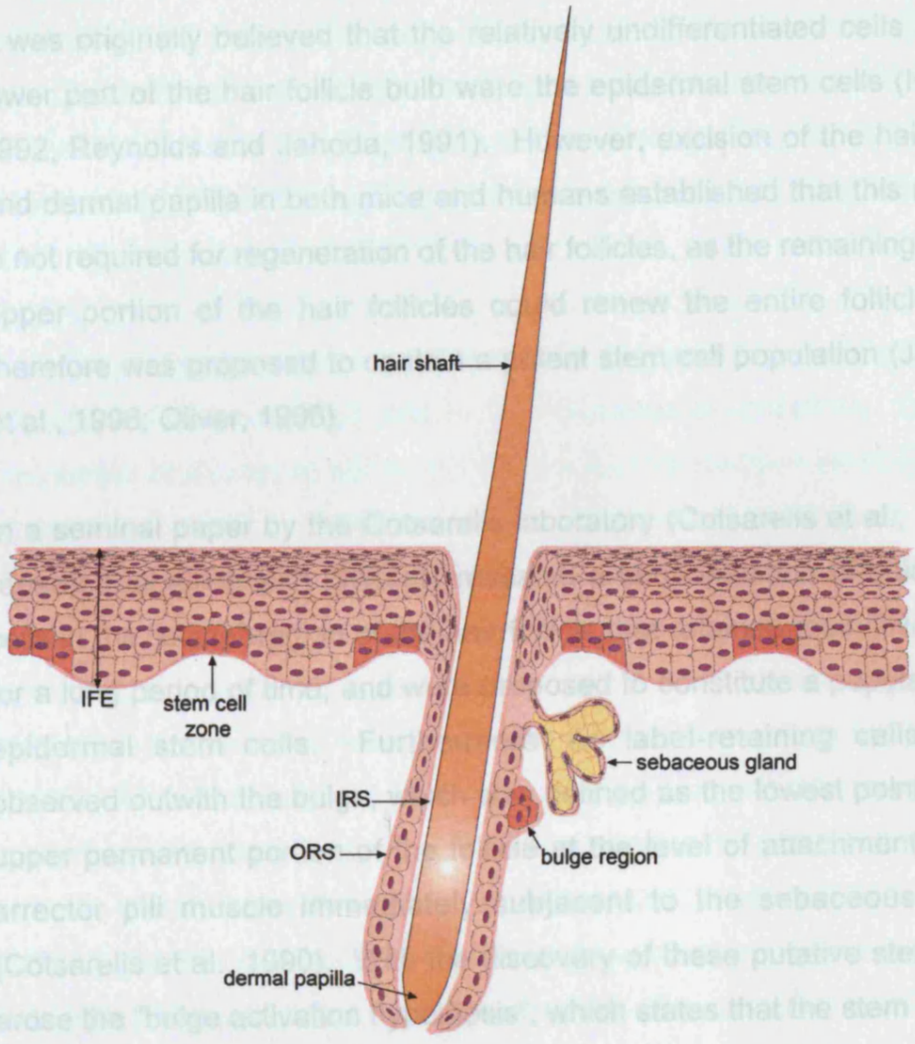

Figure 1.5) The epidermis and its postulated stem cell zones.
Stem cells are believed to be located at the top of the rete ridges in the basal layer of the interfollicular epidermis (IFE), and in the bulge region of the hair follicle beneath the sebaceous gland. The outer root sheath (ORS) is contiguous with the basal layer of the IFE and, together with the inner root sheath (IRS), forms a channel for the hair shaft. The dermal papilla is a cluster of specialised mesenchymal cells in the hair follicle bulb. Diagram courtesy of MJ Lovell.

1.8.1) Stem cells in the bulge region

It was originally believed that the relatively undifferentiated cells in the lower part of the hair follicle bulb were the epidermal stem cells (Hardy, 1992; Reynolds and Jahoda, 1991). However, excision of the hair bulb and dermal papilla in both mice and humans established that this region is not required for regeneration of the hair follicles, as the remaining intact upper portion of the hair follicles could renew the entire follicle and therefore was proposed to contain a potent stem cell population (Jahoda et al., 1996; Oliver, 1966).

In a seminal paper by the Cotsarelis laboratory (Cotsarelis et al., 1990), repeated labelling with [3H]TdR revealed a subpopulation of epidermal cells in the "bulge" region of the hair follicle that retained their DNA label for a long period of time, and were proposed to constitute a population of epidermal stem cells. Furthermore, no label-retaining cells were observed outwith the bulge, which was defined as the lowest point of the upper permanent portion of the follicle at the level of attachment of the arrector pili muscle immediately subjacent to the sebaceous gland (Cotsarelis et al., 1990). With the discovery of these putative stem cells arose the "bulge activation hypothesis", which states that the stem cells in the bulge form all epidermal lineages upon stimulation by associated mesenchymal cells (Cotsarelis et al., 1990). In support of this hypothesis, bulge cells are in contact with the mesenchymal dermal papilla of the bulb during telogen, and are the first cells to undergo proliferation at the onset of hair growth during early anagen (Wilson et al., 1994), possibly due to paracrine signalling by the cells in the dermal papilla *via* their epithelial: mesenchymal contacts.

The potential of cells in the bulge to proliferate and form new keratinocytes was confirmed in mice following BrdUrd injection twice daily for 3 days, and a subsequent 8-week chase period, as label-retaining cells were restricted to the bulge region. After this 8-week chase period all follicles were in telogen, although when the chase was extended to 10-

weeks, some follicles in the anagen growth phase were observed that contained labelled cells in their lower regions, and were therefore directly descended from cells in the bulge (Taylor et al., 2000). Further evidence for a population of stem cells in the bulge was shown by dissection of rat whisker hair follicles and evaluation of their clonogenicity *in vitro*, which revealed that 95% of clonogenic cells are located in the bulge (Kobayashi et al., 1993; Oshima et al., 2001).

A novel double-labelling technique was devised to investigate the migration of bulge-derived cells in the adult mouse epidermis. Cells of the normal adult mouse epidermis have a low proliferative index (2 - 4% of cells) and a long cell cycle time (72 – 100 hours) (Loeffler et al., 1987; Potten and Loeffler, 1987). To overcome the experimental limitations posed by a long cell cycle, epidermal wounds were created on the dorsal epidermis of 7-week-old mice when the hair follicles were in telogen, to stimulate stem cell proliferation and shorten the cell cycle (Wilson et al., 1994). The first pulse of BrdUrd was administered 21 hours post-wounding when epidermal regeneration was at its peak, followed by several chase intervals of 1 – 18 hours and a second injection of [3H]TdR. In the wounded epidermis, a 10-hour interval between BrdUrd and [3H]TdR administration resulted in the double labelling of keratinocytes in the upper regions of the follicles. Subsequent chase periods of 10, 20 and 30 hours resulted in a progressive decline in double-labelled cells of the upper follicles, and a concurrent marked increase in double-labelled cells in the IFE. These DNA-labelling studies advocate the "bulge activation hypothesis", and confirm that the progeny of stem cells in the bulge can undergo at least two independent pathways of migration and differentiation, moving downward to form cells in the lower follicle, and upward migration to form cells in the upper follicle and IFE (Taylor et al., 2000).

Barrandon and colleagues (Oshima et al., 2001) dissected fragments of bulge regions obtained from whisker (vibrissal) follicles of adult ROSA26 mice, which constitutively expressed a *lacZ* reporter gene under the

control of "SV40" promoter, and implanted them into the vibrissal follicles of wild type mice that had had their own bulge region partially amputated. Cells derived from the transplanted bulge regions were detected by immunohistochemical staining for β-galactosidase, which revealed that cells from the bulge migrate to the upper and lower regions of the hair follicles and generate all epidermal lineages of the hair follicle including the IRS, ORS, hair shaft, and the sebaceous glands. Furthermore, fragments of the ROSA26 bulge region from vibrissal follicles were implanted onto the back skin of wild type mouse embryos, and when the embryos were born, the implanted regions were then transferred onto the back of adult athymic mice to prevent graft rejection. Transplanted bulge regions produced entire hair follicles, although these follicles were both morphologically and functionally typical of back hair, or pelage, follicles (Oshima et al., 2001). Therefore, epidermal stem cells in the bulge do not appear to be pre-programmed to form definitive lineages, and instead it seems that the fate of their cell progeny is influenced by external cues from the surrounding environment i.e., the stem cell niche.

As stem cells cycle more slowly than their differentiating counterparts, and thereby retain incorporated DNA label for lengthy periods, classification of stem cells as label-retaining cells is an effective means of their identification. However, it is surprising that stem cell in the bulge appear to retain their DNA label for several weeks, as the estimated stem cell cycle time in the adult mouse epidermis is approximately 8 days (reviewed in (Potten, 2004; Potten and Booth, 2002)). Therefore, a slow cell cycle is unlikely to be the apposite explanation for the prolonged label retention of the epidermal stem cells in the bulge. The Cairn's hypothesis (Cairns, 1975), describes the selective retention of the template DNA strand by a stem cell during division, thereby protecting against DNA replication-associated errors, which are more frequent in the newly transcribed daughter DNA strand and can lead to carcinogenesis *via* the cancer stem cell hypothesis (see sections 1.1 and 1.5.2). Therefore, when new stem cells are being made i.e., during development or in tissue regeneration after wounding, DNA label would be incorporated into the

permanent template strand, which is selectively retained throughout subsequent stem cell divisions. Retention of template DNA by stem cells in the intestine has recently been confirmed in a double-labelling study (Potten et al., 2002) but, although this would account for the prolonged expression of DNA label by cells in the bulge region, has not yet been established in the epidermis.

1.8.2) The epidermal proliferative unit

Early histological analyses of the stratum corneum of the mouse dorsal epidermis revealed that this layer is assembled into discrete plate-like columns of approximately 10 cells that can be traced downward to the basal layer. It was proposed that progenitor cells in the basal layer divide and migrate upward to produce the differentiated cells of the entire column, thus compensating for cells that are shed from the skin surface during keratinisation (Mackenzie, 1970; Potten, 1974). This 10-cell basal structure was named the epidermal proliferative unit (EPU), and was proposed to consist of a single central stem cell surrounded by its transit amplifying cell progeny (Figure 1.6; (Christophers, 1971; Mackenzie, 1969; Potten, 1974)). Accordingly, subsequent irradiation and kinetic studies showed that epidermal stem cells comprise approximately 10% of the cells of the basal layer in the mouse epidermis (Clausen and Potten, 1990; Kolodka et al., 1998; Mackenzie, 1997; Potten and Morris, 1988; Withers, 1967), although other studies have suggested that only 2% (Morris et al., 1985) and 4% (Bickenbach and Chism, 1998) of the basal cells in mice are stem cells, and therefore, the exact number of stem cells in the epidermis remains indeterminate.

In the human epidermis, the structural organisation of the stratum corneum resembles that of the mouse, implying that a basal proliferative compartment similar to the mouse EPU exists, although it has not yet been possible to trace the boundaries of each column to the basal layer and thus identify a definitive stem cell zone (reviewed in (Potten, 2004; Potten and Booth, 2002)). High levels of β_1 integrin expression are

believed to be a feature of epidermal stem cells (section 1.9.1; (Jones et al., 1995; Mackenzie, 1970)). The basal cells with the highest β_1 integrin expression are localised in evenly spaced clusters within projections of the IFE into the dermis, known as rete ridges. Furthermore, keratin-19 is a potential marker of human epidermal stem cells (section 1.9.2) and is co-localised with β_1 integrin-expressing slowly-cycling cells in deep rete ridges in the human IFE (Michel et al., 1996). Therefore, although a definitive stem cell-containing, EPU-like structure has not been identified in the human epidermis, the bulk of evidence suggests that these stem cells are located within rete ridges above the mesenchymal dermal papilla.

Substantial evidence supports the notion that stem cells in the IFE are less potent and less numerous than those in the bulge (Cotsarelis et al., 1990), and that cells in the bulge have a longer lifespan than those in the IFE (Morris and Potten, 1999; Taylor et al., 2000). When cultured *in vitro*, bulge keratinocytes yield larger colonies than those from other skin sites, which has led to speculation that stem cells in the IFE may be the progeny of the bulge stem cells (Morris et al., 2004). It is well documented that the bulge stem cells are multipotent, capable of forming all the differentiated lineages of the hair follicles and the IFE. However, cells derived from the stem cells in the IFE appear to be unipotent, forming only the keratinocyte lineages found in the IFE itself (reviewed in (Alvarez-Dolado et al., 2003)).

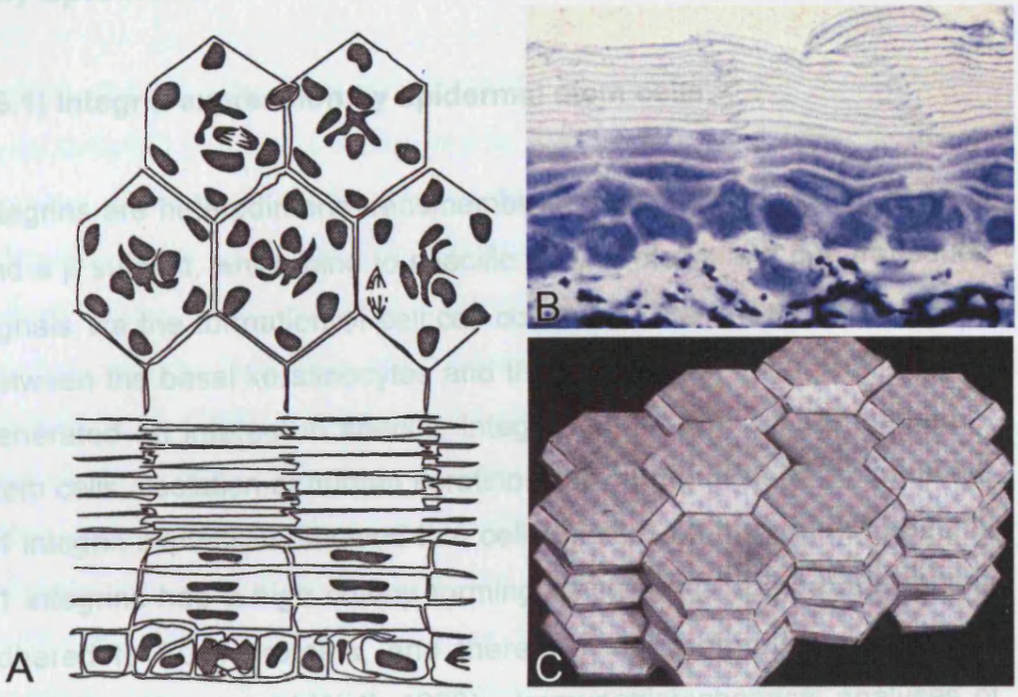

Figure 1.6) The epidermal proliferative unit (EPU).

EPUs are semi-independent units of cells, found in slowly-proliferating rodent epidermis. The stratum corneum is composed of flattened, hexagonal cells (surface view shown in upper part of A; C). The underlying stratum granulosum and stratum spinosum layers are aligned into columns, which project downward and encompass approximately 10 cells in the basal layer (lower part of A; B). Mitotic cells are located at the edges of the basal layer, and are believed to derive from a central basal stem cell(s) and undergo lateral and then suprabasal migration throughout each epidermal layer until they are shed at the skin surface. (A, from (Leigh et al., 1994); B, C from (Wright and Alison, 1984)).

1.9) Epidermal stem cell markers

1.9.1) Integrin expression by epidermal stem cells

Integrins are heterodimeric transmembrane receptors consisting of an α and a β subunit, which bind to specific ECM proteins and can transduce signals *via* the formation of cell:cell contacts. The known associations between the basal keratinocytes and their underlying basal lamina have generated an interest in specific integrin expression by the epidermal stem cells. Isolation of human keratinocytes on the basis of their surface β1 integrin expression showed that cells expressing the highest levels of β1 integrins had a high colony forming efficiency in culture and rapidly adhered to ECM proteins, and therefore may comprise a stem cell population (Jones and Watt, 1993). Immunohistochemical analyses of human skin showed that β1 integrin expression is restricted to discrete patches of cells in the basal layer, and differentiating cells located suprabasally do not express β1 integrins (Jones et al., 1995). However, high β1 integrin expression was observed in approximately 20 – 45% of basal cells, which is at least double the proportion that are estimated to be stem cells *in vivo* (Jones et al., 1995).

The β1 integrin gene knockout mouse is early embryonic lethal. Therefore, to investigate the consequences of epidermal-specific β1 integrin deletion, mice with floxed β1 alleles were crossed with mice expressing the Cre-recombinase enzyme under the control of either a keratin-14 or keratin-5 promoter, both of which proteins are constitutively expressed in basal keratinocytes. These animals show severe defects in basement membrane assembly and organisation and have impaired keratinocyte proliferation and ineffective cell migration, although proliferation and terminal differentiation of keratinocytes does occur (Brakebusch et al., 2000; Grose et al., 2002; Raghavan et al., 2000). It appears that β1 integrin is essential for the migration of keratinocytes,

possibly *via* interactions with the basement membrane, although it is not essential for keratinocyte proliferation and differentiation.

Studies of a β1 integrin-deficient embryonic stem cell line showed that these cells fail to form keratinocytes *in vitro*, although will differentiate to produce keratinocytes *in vivo* when allowed to form teratomas by subcutaneous injection in mice, and following injection into the skin of β1-null/wild type chimeric mice. Additionally, ECM assembly was impaired in ES cell cultures, but not in the teratomas or chimeric mouse epidermis. (Bagutti et al., 1996). It was initially proposed that a lack of regulatory signals from the basement membrane in the culture environment prevents keratinocyte differentiation (Bagutti et al., 1996). This is fitting with the theory that stem cells are regulated by signals from their underlying niche, which is believed to comprise the underlying mesenchymal cells and their associated basement membrane factors (section 1.2). However, keratinocyte differentiation was not induced in co-cultures of β1 integrin-deficient ES cells with epidermal basement membrane factors (Bagutti et al., 2001).

A later study has also highlighted α6 integrin as a stem cell marker in the human epidermis. Approximately 10% of basal epidermal cells express α6 integrin and exhibit an enhanced regenerative capacity (Li et al., 1998). However, the use of α6 integrin in identifying epidermal stem cells remains controversial, as previous studies found little correlation between the level of α6β4 integrin expression and proliferative potential (Jones et al., 1995; Jones and Watt, 1993). Additionally, mice lacking the α6β4 integrin heterodimer display severe epidermal blistering, but do not demonstrate reduced proliferation or differentiation of keratinocytes (DiPersio et al., 2000).

1.9.2) Keratin protein expression by epidermal stem cells

The keratins are a multigene family of intermediate filament proteins that are differentially expressed in specific epithelial tissues, including the skin. In the epidermis, keratin (K) 5 and K14 are generally found in the undifferentiated cells of the basal layer, and K1 and K10 are found suprabasally in more differentiated keratinocytes (Bailleul et al., 1990; Byrne and Fuchs, 1993; Ramirez et al., 1994; Vassar et al., 1989). The switch in keratin expression between the proliferating and differentiating compartments of the epidermis indicates that changes in keratin filament organisation, and the ensuing changes in cellular adhesion may influence keratinocyte function (reviewed in (Fuchs, 1990)).

Unlike K14, which is ubiquitously expressed by all cells in the basal epidermis, K15 is restricted to patches of label-retaining cells in this layer in the human hair follicle bulge region, and thus K15 has been nominated as a marker of human epidermal stem cells (Lyle et al., 1998; Porter et al., 2000; Waseem et al., 1999). Moreover, label-retaining cells in the bulge have recently been shown to co-express both K15, and the primitive stem cell marker, CD34 (Trempus et al., 2003).

Liu and colleagues (Liu et al., 2003) cloned the promoter region of the mouse *K15* gene with a downstream *lacZ* reporter gene, and generated a K15/lacZ transgenic reporter mouse in which K15 activity was detected by β-galactosidase expression. Transgenic mice were injected with [3H]TdR twice a day for 3 days, followed by a chase period of 50 days, to label DNA in slowly cycling cells. In adult transgenic mice, lacZ expression was strikingly localised to label-retaining bulge cells throughout all stages of the hair cycle (Liu et al., 2003). As a continuation to these results, a second transgenic mouse was produced that expressed CrePR1 fusion protein, under control of the K15 promoter (*Ktr1-15-CrePR1)*. CrePR1 consists of a Cre-recombinase enzyme and a truncated progesterone receptor that binds the progesterone antagonist, RU486. In *Ktr1-15-CrePR1* mice, CrePR1 is inactive in K15-positive cells except during RU486 treatment, wherein CrePR1 enters the nucleus and causes recombination. *Ktr1-15-CrePR1* mice were further crossed with

R26R mice to produce *Ktr1-15-CrePR1;R26R* transgenic mice, which ubiquitously express lacZ under the control of the ROSA26 promoter following Cre-mediated removal of an inactivation sequence. Treatment of *Ktr1-15-CrePR1;R26R* mice with RU486 resulted in permanent expression of lacZ in the bulge region, and also in their progeny, thus demonstrating that bulge stem cells are K15-positive, have a high proliferative potential and are long-lived. These studies unequivocally demonstrate that bulge stem cells are multipotent and can form three differentiated epidermal lineages, as lacZ-labelled cells were seen in hair follicles, IFE and in the sebaceous glands, although the major flux of cells appeared to contribute to generation of the new hair follicle during the hair cycle, and it is postulated that separate populations of stem cells exist within the IFE and sebaceous glands (Morris et al., 2004). In the same study, the K15 promoter region was cloned upstream of *eGFP* and transgenic mice were created wherein the K15/eGFP-positive cells in the bulge could be isolated by fluorescence-activated cell sorting (FACS). These cells possessed several characteristics of epidermal stem cells as they co-expressed α6 and β1 integrins and CD34, and they also had a high proliferative potential and formed large colonies *in vitro*. Furthermore, the multipotency of these cells was confirmed as isolated stem cells can regenerate the entire epithelium when combined with neonatal mouse dermal cells in culture (Morris et al., 2004).

The aforementioned study by the laboratory of Cotsarelis (Morris et al., 2004), is one of two recent papers that have made a considerable advancement in the isolation of a multipotent epidermal stem cell. The second paper comes from the Fuchs laboratory, and outlines the transcriptional profile of a population of label-retaining cells isolated from the stem cell niche (Tumbar et al., 2004). In this study, transgenic mice were engineered to express histone H2B-green fluorescent protein (GFP) controlled by a tetracycline (tet)-responsive regulatory element (TRE), and were further crossed with mice expressing a K5 promoter, driven by the tet repressor, VP16. Administration of tet induced K5 expression in the epidermis, and in the absence of tet the epidermis was GFP-positive.

Four-week-old mice were administered tet for 4 weeks, and following a 4 month chase period, less than 1% of bulge cells retained GFP fluorescence i.e., had not differentiated to express K5. At the onset of anagen, GFP-positive cells initiate new follicle formation and migrate downward from the bulge to produce multiple epidermal lineages including cells in the IRS, ORS, matrix and hair shaft. The GFP-positive cells also repopulated the wounded epidermis *in vivo*, and had high proliferative capacity *in vitro*. The authors confirm the co-expression of epidermal stem cell markers in the bulge including, K15, K19, α6 and β1 integrins, and CD34, and defined the mRNA expression profile of these cells which interestingly revealed expression of three genes that are also common to HSCs, ES cells and neural stem cells, namely *Eps8, Col18a1, Pkd2* (Tumbar et al., 2004).

K19 is another keratin protein that has been proposed as a stem cell marker, although recent evidence suggests that K15 is perhaps a more convincing candidate (Morris et al., 2004; Tumbar et al., 2004). K19 co-localises with the label-retaining cells in the bulge region of hair follicles in adult human skin (Lane et al., 1991; Stasiak et al., 1989). K19 expression within label-retaining cells in the skin was confirmed in a later study combining [3H]TdR labelling with K19 immunodetection, although K19 was not expressed on all label-retaining cells and therefore may not be present in all stem cells (Michel et al., 1996). K19-positive cells also co-express high levels of the putative epidermal stem cell marker $\alpha_3\beta_1$ integrin, strongly implicating K19 as a marker of epidermal stem cells (Michel et al., 1996). Interestingly, the numbers of K19-positive cells appeared to decrease with age, suggesting a decline in stem cell number from birth to adulthood (Michel et al., 1996).

1.9.3) *p63* expression by epidermal stem cells

Another factor associated with epidermal stem cells is the transcription factor *p63*, a homologue of the tumour suppressor gene *p53* (Lohrum and

Vousden, 2000). Mice with a targeted deletion of the *p63* gene locus have major defects in their limb and craniofacial development, as well as a striking absence of stratified epithelia (Celli et al., 1999; Yang et al., 1999). Immunohistological analyses of human skin revealed that p63 protein expression was restricted to clusters of slow-cycling, undifferentiated cells in the basal epidermis, implicating *p63* as a marker of distinction between epidermal stem cells and their transit amplifying progeny (Pellegrini et al., 2001; Yang et al., 1998).

1.9.4) Melanoma-associated chondroitin sulfate expression by epidermal stem cells

Keratinocytes synthesise a number of proteoglycans that are differentially expressed depending on the proliferative status of the cell. Melanoma-associated chondroitin sulfate (MCSP) is a high molecular weight proteoglycan that is highly expressed in human melanomas and in cultured melanoma cell lines (Pluschke et al., 1996). MCSP has a restricted pattern of expression in small clusters of basal cells in the human IFE, and is co-localised with β_1 integrin in these cells (Kupsch et al., 1995). In the human hair follicles, MSCP expression is relatively widespread in the ORS, although co-expression of both MCSP and K15 is restricted to basal cells in the centre of the ORS (Kupsch et al., 1995). Moreover, MCSP is co-localised with other putative epidermal stem cell markers, keratin-19, β_1 and α6 integrins, within a subset of cells in this region of the ORS (Ghali et al., 2004). These data suggests that MCSP, when co-expressed with other specific antigens, may provide a good marker of human epidermal stem cells in both the IFE and the bulge region of the hair follicles.

1.10) The epidermal stem cell niche

It is generally accepted that a population of stem cells exists in the bulge region of the hair follicles and in specialised clusters in the IFE, although the stem cell niches for these stem cells are as yet undefined and consequently the mechanisms that regulate epidermal stem cell behaviour are unclear. The bulge activation hypothesis states that signals from mesenchymal cells in the dermal papilla of the hair bulb stimulate the multipotent differentiation of the keratinocyte stem cell (section 1.8.1). During catagen, the lower two-thirds of the hair follicle regress and the dermal papilla moves upward to the level of the bulge, where it remains throughout telogen. When a new hair cycle is initiated with the onset of anagen, cells derived from the bulge move downward and proliferate and differentiate to form a new hair shaft, and other bulge cell derivatives move upward to maintain the upper follicle and sebaceous gland and to replenish cells in the IFE. Therefore, the dermal papilla appears to be the most likely component of the epidermal stem cell niche, and mesenchymal: epithelial interaction between the cells in the dermal papilla and in the bulge are responsible for stimulating the stem cells to divide and leave the niche and undergo differentiation. Although the bulge activation hypothesis was established based upon circumstantial evidence, a number of studies have shown that proliferation and differentiation of epidermal cells is dependent on direct contact between the epithelial bulge and the mesenchymal dermal papilla. For example, if the dermal papilla is mechanically separated from the growing hair follicle, follicle growth ceases (Link et al., 1990). Moreover, mice that do not express bone morphogenetic protein-4 (BMP-4), which is normally produced in the dermal papilla cells, show complete loss of all hair due to reduced or absent keratinocyte differentiation and dysregulated cell proliferation (Kulessa et al., 2000).

1.11) Signalling pathways of epidermal cell differentiation

Signals that initiate and regulate the defined pathways of epidermal stem cell differentiation are not clear. However, recent advances have been made to identify the molecules and pathways that regulate stem cell fate.

1.11.1) The Wnt/β-catenin signalling pathway

As described in section 1.6.1, members of the T cell factor/lymphoid-enhancing factor (Tcf/LEF) family of DNA-binding proteins are transcriptional regulator molecules, which are bound by β-catenin in response to canonical Wnt signalling, and activate the transcription of genes that induce cell proliferation. In the epidermis, β-catenin and Tcf/LEF family members are expressed in both mesenchymal cells of the dermal papilla and in keratinocytes (Alonso and Fuchs, 2003; DasGupta and Fuchs, 1999; Zhou et al., 1995). Mice with a homozygous germ-line mutation in the *LEF-1* gene showed sparse hair growth and complete loss of whisker follicles (van Genderen et al., 1994), and transgenic mice that mis-express *LEF-1* display striking abnormalities in their hair follicle orientation and patterning during development (Zhou et al., 1995). Transgenic mice that lack β-catenin binding site expression in basal keratinocytes showed progressive hair loss and formed dermal cysts derived from cells in the base of the hair follicles. These cysts expressed markers of differentiation common to cells in the IFE and thus β-catenin appears to regulate keratinocyte differentiation into specific lineages (Niemann and Watt, 2002). Transgenic mice that express a truncated stabilised form of β-catenin under the control of an epidermal keratin promoter display signs of *de novo* hair follicle morphogenesis. These hair follicles induce *LEF-1*, causing hyperproliferation of keratinocytes (Gat et al., 1998). Therefore, β-catenin appears to regulate keratinocyte stem cell proliferation *via* regulation of *LEF-1* gene transcription.

1.11.2) *c-Myc* regulation of epidermal stem cell function

The *c-Myc* oncogene is a downstream target of the β-catenin/Tcf/LEF pathway, and can suppress the proliferation of normal human epidermal stem cells in culture, and stimulate pathways of terminal differentiation in these cells (Gandarillas and Watt, 1997). To study the function of *c-Myc in vivo*, an inducible system was generated in which *c-Myc* was fused to the oestrogen receptor (c-MycER), and was then targeted to the basal keratinocytes using the K14 promoter. In this system, *c-Myc* is held in an inactive state until the addition of 4-hydroxytamoxifen (4-OHT). The effects of *c-Myc* on keratinocytes *in vivo,* were not consistent with the *in vitro* observations described above. In heterozygous transgenic mice, 4-OHT treatment stimulated proliferation of cells in the IFE, leading to a thickened epidermis and an increased number of differentiated cell layers. However, the hair follicles showed an abnormal morphology and contained increased numbers of sebocytes. Therefore, *c-Myc* appeared to promote sebocyte differentiation at the expense of hair differentiation (Arnold and Watt, 2001). In a similar study, expression of *c-Myc* in the basal keratinocytes produced a 50% decrease in β1 integrin-expressing label-retaining cells, concomitant with a loss of hair in these animals (Waikel et al., 2001). These data suggest that *c-Myc* activation depletes the epidermal stem cell population in the hair follicles, possibly by down-regulation of β1 integrins, which in turn impairs keratinocyte migration.

1.11.3) Bone morphogenetic proteins

The bone morphogenetic proteins (BMPs), part of the TGFβ superfamily, have known roles in the regulation of cell proliferation, differentiation and apoptosis in many tissues, including skin (reviewed in (Massague and Chen, 2000)). BMPs activate signal transduction *via* their type I (BMPR-IA and BMPR-IB) and type II (BMPR-II) serine/threonine kinase receptors. A number of BMP antagonists have been described, including Noggin, which binds BMP-2 and BMP-4 with a high affinity, and binds

BMP-7 with a lower affinity (reviewed in (Botchkarev, 2003)). Upon BMP binding to its receptor, specific Smad proteins are phosphorylated and translocate to the nucleus to regulate transcription of BMP target genes. BMP signalling is believed to be vital for hair follicle induction, and BMP-2 and BMPR-IA are present in the epithelial cells of the hair follicle, and BMP-4 is expressed by the cells in the dermal papilla of mice (Botchkarev et al., 1999; Lyons et al., 1989; St-Jacques et al., 1998). In mice with a targeted deletion of the BMP-neutralisation protein Noggin, hair growth is significantly retarded (Botchkarev et al., 1999). It is proposed that Noggin neutralises the inhibitory action of BMP-4 on anagen, and stimulates hair growth by upregulating the transcriptional repressor molecule LEF1, which is supported by the observed active hair growth in mice *in vivo* following administration of Noggin protein (Botchkarev et al., 2001). Therefore, it appears that cross-talk between the BMP and Wnt/β-catenin signalling pathways are critical for the normal hair cycle to occur. Interactions between the Wnt and BMP signalling pathways were recently confirmed in mice with a Cre-mediated mutation of BMPR-IA. These mice had a lack of external hair and the IRS failed to differentiate. Nuclear β-catenin was absent from the epithelium of severely mutated hair follicles. These data suggest that activation of the Wnt pathway is a downstream effect of BMPR-IA signalling, both of which are essential for normal differentiation and proliferation of postnatal hair follicles (Andl et al., 2004). The downstream target molecules of BMP signalling are not yet fully understood, although the transcription factor Msx-2 is expressed on hair matrix keratinocytes and mediates the effects of BMP on LEF-1, Foxn1 and Hoxc13 transcription factors, which in turn regulate the expression of keratin genes by keratinocytes (reviewed in (Botchkarev, 2003; Fuchs, 2001)).

1.11.4) Sonic hedgehog

The sonic hedgehog (Shh) signalling cascade is one of the key pathways in development of the epidermis and hair follicles and in skin

tumourigenesis. The transmembrane receptor Patched (Ptc), is activated by Shh and triggers a signalling cascade modulated by Smoothened (Smo), which in turn elicits nuclear translocation of the transcription factor Gli and the transcription of downstream target genes, including members of the Wnt and TGFβ family (Ingham, 1998; Ingram et al., 2002). Shh is expressed in the developing epidermis and hair follicles, and Ptc expression is in the adjacent mesenchymal cells (Bitgood and McMahon, 1995; Iseki et al., 1996; Motoyama et al., 1998; Oro et al., 1997). The anagen-inducing properties of Noggin are mediated, at least partly by Shh signalling in the hair follicle. Shh is upregulated in the hair follicle following Noggin administration, whereas BMP-4 downregulates Shh expression (Botchkarev et al., 2001). In Shh knockout mice formation of follicles, hair shafts and sebaceous glands was inhibited, although the epithelial cells undergo terminal differentiation to keratinocytes. $Shh^{-/-}$ mice lacked a dermal papilla, demonstrating a paracrine regulation of development of the mesenchymal components of the hair follicle by Shh in the epidermis (Chiang et al., 1999). Therefore, Shh regulates the development of the mesenchymal components of the hair follicle, and the absence of dermal papillae in Shh-deficient mice prevents hair follicle morphogenesis. The differentiation of keratinocytes in the absence of the stem cell niche, supports the "stem cell niche" hypothesis (Schofield, 1978).

The Wnt, TGFβ, and Shh signalling pathways appear to reciprocally modulate the epidermal stem cells, with some overlap in each pathway. For example, normal signalling *via* both the Wnt and BMP pathways are vital for induction of follicle growth and the terminal differentiation of keratinocytes, whereas the Shh pathway is vital for the proliferative expansion of the hair follicles, but is not required during hair lineage differentiation. The BMP signalling pathway is also required to induce hair growth, and is a probably candidate for a downstream target of the Wnt pathway. Moreover, ectopic differentiation of sebocytes was observed in separate studies where either the Shh pathway (Allen et al.,

2003), or the BMP pathway (Guha et al., 2004) was dysregulated, suggesting that both pathways are essential for normal lineage differentiation in the sebaceous glands.

1.12) Bone marrow stem cells

1.12.1) Haematopoietic stem cells

The pluripotent haematopoietic stem cell (HSC) can reconstitute the entire cell repertoire of the blood (Lagasse et al., 2001; Morrison and Weissman, 1994; Weissman, 2000), and is one of the few adult stem cells for which definitive markers have been identified. HSCs are most commonly isolated by FACS using specific antibodies to cell surface proteins e.g., all adult HSCs express c-Kit and Sca-1, though lack, or express very low levels of markers of lineage differentiation ($Lin^{-/lo}$) (Uchida and Weissman, 1992). Specific cell surface proteins are gradually expressed as HSCs mature and their self-renewal potential becomes limited, and known markers of lineage differentiation in HSCs include Thy-1, Flk2, B lymphocytes (CD45), T lymphocytes (CD3), granulocytes (Ly-6G), monocytes (CD11) and erythrocytes (TER-119) (reviewed in (Kondo et al., 2003)). The sialomucin CD34 was originally identified as the definitive HSC marker, although CD34 expression appears to fluctuate as subpopulations of CD34-positive (+) and CD34-negative (-) HSCs have been identified in both human and mouse bone marrow (BM), (reviewed in (Kondo et al., 2003)). The glycoprotein AC133 (CD133) may be a more primitive marker of human HSCs, as it is expressed on both CD34+ and CD34- BM fractions and is down-regulated as HSCs differentiate, and virtually all mature haematopoietic lineages lack CD133 expression (Gallacher et al., 2000; Yin et al., 1997). Human-derived CD133+ cells have been shown to repopulate the sheep BM (Yin et al., 1997).

1.12.2) Side populations cells

Both murine and human BM express a highly-enriched HSC population, the side population (SP) cells, which are identified by their ability to efflux

the DNA binding dye Hoechst 33342 on flow cytometric analysis. Murine SP cells represent approximately 0.1% of the whole BM, although can reconstitute the entire haematopoietic system in lethally irradiated recipients (Goodell et al., 1996). Recent analyses of CD34- and CD34+ subpopulations of murine SP cells has revealed that CD34- SP cells contain the greatest enrichment of primitive stem cells (Parmar et al., 2003).

1.12.3) Mesenchymal stem cells

Mesenchymal stem cells (MSCs) in the adult BM support haematopoiesis *via* cell signalling and the secretion of specific growth factors and cytokines (Cherry et al., 1994; Guerriero et al., 1997; Moreau et al., 1993), and are progenitor cells for each of the differentiated lineages found in the connective tissue such as bone, fat, muscle, tendon and cartilage (reviewed in (Barry and Murphy, 2004; Roufosse et al., 2004). MSCs comprise approximately 0.001 – 0.01% of the total number of nucleated cells in the BM, although, similar to HSCs, can be enriched *in vitro*. MSCs were first isolated from the haematopoietic component of rat BM in 1966, using standard cell culture techniques (Friedenstein et al., 1966), and it is now well documented that MSCs present *in vitro* as fibroblastic-like, adherent cells capable of extended proliferation. Attempts to characterise the cell surface antigens expressed by MSCs has proven difficult due to the low numbers of MSCs in adult BM. In the mouse, CD34+, CD45+, CD11b+ fractions of BM are believed to be enriched for MSCs (Baddoo et al., 2003; Ortiz et al., 2003). In the human BM, CD34 (Simmons and Torok-Storb, 1991a), CD166 (activated leukocyte-cell adhesion molecule) (Bruder et al., 1998), CD105 (transforming growth factor receptor, endoglin) (Barry et al., 1999), CD73 (Barry et al., 2001) and the stromal progenitor (Stro-1) antigen (Simmons and Torok-Storb, 1991b) are all expressed on MSCs, although are not MSC-specific, and thereby do not qualify as definitive stem cell markers (reviewed in (Barry and Murphy, 2004)).

1.12.4) Multipotent adult progenitor cells

A subpopulation of MSCs, the multipotent adult progenitor cell (MAPC), has been isolated from the BM of both rodents (Jiang et al., 2002) and humans (Reyes et al., 2001). MAPCs were initially separated by their negative expression of the haematopoietic markers, CD45 and glycophorin-A (GlyA). Human MAPCs are characteristic of classical MSCs as they lack expression of CD44, major histocompatibility complex (MHC) class I, and β2-microglobulin (Pittenger et al., 1999), and also do not express the HSC markers CD34, CD45 and c-Kit. Murine MAPCs have a similar phenotype to the human MAPC as they do not express CD34, CD44, CD45, c-Kit, MHC class I and II, have a low level of expression of Flk-1, Sca-1 and Thy-1, and express high levels of CD13 and stage-specific antigen I (SSEA-1). The morphology and phenotype of MAPCs was stable for over 50 population doublings in humans (Reyes et al., 2001), and 120 population doublings in the mouse (Jiang et al., 2002). Both human and mouse MAPCs can differentiate to form all mesenchymal cell lineages and endothelial lineages, and mouse MAPCs can contribute to the epithelium of the liver, lung and gut (Jiang et al., 2002). The MAPCs are similar to embryonic (ES) stem cells in their phenotype, nature and differentiation potential (Jiang et al., 2002), although MAPCs are readily accessed from BM and thus do not carry any ethical stigma. Furthermore, ES-derived cells often develop into teratomas (Thomson et al., 1998), whereas MAPC progeny do not (Jiang et al., 2002).

1.12.5) Endothelial progenitor cells

Vasculogenesis is the *de novo* formation of blood vessels from vascular precursor cells, or angioblasts, originating in the primitive mesoderm (Risau and Flamme, 1995). Angioblasts give rise to endothelial cell channels, which form the lining of the blood vessels and are surrounded by a layer of smooth muscle "mural" cells, although to date a mural cell progenitor cell has not been identified. Traditionally, vasculogenesis was

believed to be confined to early embryogenesis, and postnatal blood vessel formation was attributed to "angiogenesis", wherein fully-differentiated endothelial cells in pre-existing blood vessels detach from the vessel wall, and migrate and proliferate to remodel the existing primary vascular network e.g., within injured vasculature and in tumour formation (Folkman, 1992). However, accumulating evidence suggests that circulating cells exist in the adult BM and peripheral blood that can form new blood vessels by neovasculogenesis, leading to the novel paradigm of "postnatal vasculogenesis".

A population of circulating endothelial precursor cells (EPCs) was initially isolated in 1963 by the endothelialisation of Dacron grafts by cells in the peripheral blood (Stump et al., 1963). EPCs were first characterised based on their expression of vascular endothelial growth factor receptor-2 (VEGFR-2, Flk-1 in mouse) and CD34, which are also expressed by angioblasts and HSCs (Asahara et al., 1997; Shi et al., 1998). These BM-derived EPCs have a high proliferative capacity, form homogenous monolayers *in vitro* with typical "cobblestone" cell morphology, produce nitric oxide, incorporate acetylated low-density lipoprotein (Ac-LDL) and express several endothelial markers including, von Willebrand factor (vWF), VEGFR-2, CD31 (PECAM), CD105, E-selectin and vascular cell adhesion moleulce-1 (V-CAM-1) (Asahara et al., 1997; Quirici et al., 2001; Shi et al., 1998).

However, distinction of EPCs from mature endothelial cells may be complicated by the expression by both cell types of several antigens including, VEGFR-2 (Flk-1) (Flamme et al., 1995), Tie-1 (Sato et al., 1993; Sato et al., 1995), Tie-2 (Schnurch and Risau, 1993) and vascular endothelial (VE)-cadherin (Ali et al., 1997; Peichev et al., 2000; Vittet et al., 1996). Furthermore, EPCs share a common expression of a number of HSC markers including VEGFR-1, CD34, platelet endothelial cell adhesion molecule (PECAM), Tie-1, Tie-2 and vWF, and both cell types can incorporate Ac-LDL (reviewed in (Peichev et al., 2000)). Human EPCs have recently been shown to express CD133 (Peichev et al.,

2000), which is suggested to be a primitive HSC marker (Gallacher et al., 2000; Yin et al., 1997).

Recent studies have shown that CD14+/CD34- human mononuclear cells, which are phenotypically distinguishable from MSCs and HSCs, express endothelial cell-specific markers *in vitro* including, vWF, VEGFR-1, VE-cadherin, CD105, CD31, Tie2 and endoglin (Fernandez Pujol et al., 2000; Fujiyama et al., 2003). Additionally, these CD14+ cells form functional endothelial cells in ischaemic models (Fujiyama et al., 2003; Harraz et al., 2001). It is therefore possible that the CD34-/CD14+ fraction of BM contains a potent source of EPCs (Fujiyama et al., 2003).

It is now well documented that EPCs in the mouse and human BM play an important role in vasculogenesis in both mouse and human. The isolated subpopulations of EPCs, and their contribution to vasculogenesis in specific models of injury or disease is summarised in Table 1.1.

Table 1.1) Examples of the contribution of BM-derived cells to vascular lineages in the mouse and human.

Transplanted cell phenotype	Damage or disease model	Result	Reference
CD34+, Flk-1+ cells (human)	Hindlimb ischaemia (mouse)	Neovasculogenesis, BM-derived endothelium	(Asahara et al., 1997)
Low-density BM mononuclear cells (mouse)	Tumour implant, epidermal wounding, hindlimb ischaemia, myocardial ischaemia (mouse)	Neovasculogenesis, BM-derived endothelium	(Asahara et al., 1999)
CD34+ cells (G-CSF-stimulated; human)	Myocardial infarction (mouse)	Neovasculogenesis and angiogenesis	(Kocher et al., 2001)
Lin-, c-kit+ cells (mouse)	Myocardial infarction (mouse)	Endothelial cells, smooth muscle cells	(Orlic, 2002; Orlic et al., 2001a)
CD34+, CD45+, CD133+ cells (human)	Myocardial infarction (human)	Myocardial regeneration	(Assmus et al., 2002)
CD14+, CD34- (human)	De-endothelialisation of carotid artery	Neovasculogenesis, BM-derived endothelium	(Fujiyama et al., 2003)
Peripheral blood mononuclear cells (human)	Ischaemic hindlimb (mouse)	Neovasculogenesis	(Iwaguro et al., 2002)
Lin-, c-kit+, Sca-1+ (mouse)	Atherosclerosis (mouse)	Neovasculogenesis, endothelial cells, smooth muscle cells	(Sata et al., 2002)
Lin- HSCs (mouse or human)	Retinal degeneration (mouse)	Endothelial cells in recovered retinal vasculature	(Otani et al., 2004; Otani et al., 2002)

HSCs (mouse)	Choroidal neovascularisation (mouse)	Neovasculogenesis, BM-derived endothelium	(Grant et al., 2002; Sengupta et al., 2003)
CD133+ (including CD34- subpopulation; human)	Myocardial infarction (human)	Enhanced ventricular function, angiogenesis	(Stamm et al., 2003)
SP cells (CD34-/low, c-kit+, Sca-1+; mouse)	Myocardial infarction (mouse)	Endothelial cells in small vessels	(Jackson et al., 2001)
BM (mouse)	Cerebral infarction (mouse)	Neovasculogenesis, BM-derived endothelium	(Hess et al., 2002)
HSC (Lin-, CD34-, c-kit+, Sca-1+; mouse)	Cerebral infarction (mouse)	BM-derived perivascular cells	(Hess et al., 2004)
BM (mouse)	Choroidal neovascularisation (mouse)	Neovasculogenesis, BM-derived endothelium	(Tomita et al., 2004)
HSCs(c-kit+, Sca-1+, Lin-; mouse)	N/A	BM-derived endothelium (liver, lung, heart, skeletal muscle and intestine)	(Bailey et al., 2004)
CD117+ cells (mouse)	Hindlimb ischaemia (mouse)	Neovasculogenesis, BM-derived endothelium	(Li et al., 2003)
BM (mouse)	Choroidal neovascularisation (mouse)	BM-derived endothelial cells and smooth muscle-like cells	(Espinosa-Heidmann et al., 2003)
Sca-1+, Flk-1+, c-kit+ cells (mouse)-treated with HGF	Emphysema (mouse)	BM-derived endothelial cells, pulmonary regeneration	(Ishizawa et al., 2004)
Lin- HSCs and whole BM (mouse)	β-cell injury (mouse pancreas)	Neovasculogenesis, BM-derived endothelium	(Mathews et al., 2004)

Lin-, c-kit+, Sca-1+ HSCs, or whole BM (mouse)	Choroidal neovascularisation (mouse)	Neovasculogenesis, BM-derived endothelium	(Takahashi et al., 2004)
BM (mouse)	Choroidal neovascularisation (mouse)	Neovasculogenesis, BM-derived endothelium	(Csaky et al., 2004)

1.13) Adult Stem Cell Plasticity

An expanse of evidence regarding the surprisingly flexible potential of adult stem cells in their differentiation repertoires has recently challenged the classical belief that tissue stem cells are restricted to the production of adult lineages within their tissue of residence. Consequent to this discovery, stem cells now represent an entire new field of regenerative medicine, and may hold the key to the treatment of a number of diseases such as cancer, cardiovascular disease, neurodegenerative disease and diabetes. Recently, a vast number of reports have emerged showing that adult stem cells retain a large degree of plasticity and can differentiate to form many functional adult cells within extraneous tissues. Much of this research has focussed on the adult BM as an easily accessible source of cells that have the potential to cross lineage boundaries, and transplanted adult BM cells have been shown to form adult cell types in many different tissues including the liver, kidney, heart, CNS, gastrointestinal tract, lung, and skin (reviewed in (Poulsom et al., 2002); see also Table 1.2). These pathways can be bi-directional as muscle (Jackson et al., 1999) and neuronal stem cells (Bjornson et al., 1999) can also form BM, though the latter pathway is disputed by some (Morshead et al., 2002).

It appears that selection pressure induced by target organ damage can intensify the efficacy of this process e.g., increased numbers of cells of BM origin were observed in murine models of cerebral infarction (Hess et al., 2002), myocardial infarction (Jackson et al., 2001; Orlic, 2002; Orlic et al., 2001b), and in gastrointestinal epithelia of BM-transplanted humans with graft-versus-host-disease (GvHD) or intestinal ulceration (Okamoto et al., 2002). It is proposed that BM cells respond to specific signals from damaged or diseased tissues whereupon they migrate to, and engraft within this tissue, and aid regeneration and remodelling by contributing to differentiated adult cells.

It is important to demonstrate that BM-derived adult cells are functional with a capacity to restore diseased or damaged tissues, and thus convey a possible clinical relevance. The fumarylaceto-acetate hydrolase-deficient ($Fah^{-/-}$) mouse model of the human metabolic liver disease, Type 1 tyrosinaemia, was used to confirm the functional significance of BM cell plasticity (Lagasse et al., 2000). $Fah^{-/-}$ mice were transplanted with as few as 50 purified HSCs, which travelled to the liver and restored enzyme levels to normal by forming Fah-expressing hepatocytes, thereby rescuing the mice from their fatal deficiency (Lagasse et al., 2000). A recent publication also showed amelioration of disease by BM cells, as Cu/Zn superoxide dismutase (SOD1) mice, which develop a lethal neurodegenerative disease similar to amyotrophic lateral sclerosis (ALS) in humans, show a considerable delay in the onset of ALS and have a significantly extended lifespan following BM transplant (Corti et al., 2004). Fully differentiated neurons, skeletal muscle fibres and cardiomyocytes of BM origin were observed in these mice (Corti et al., 2004). BM cells have also been shown to produce functionally competent endocrine β-cells in the pancreatic islets of mice, demonstrating a potential cell-based therapy for diabetes mellitus (Ianus et al., 2003).

1.13.1) BM contribution to the cells in the adult intestine

There are a growing number of reports that BM cells can repopulate both epithelial and mesenchymal lineages in the gastrointestinal tract of animals and man. A seminal paper by Krause and colleagues (Krause et al., 2001) demonstrated the remarkable plasticity of a single purified BM cell. Eleven months after injection of a male mouse HSC and 2×10^4 supporting female haematopoietic progenitor cells into a female mouse, multiple tissues were analysed for the presence of the Y chromosome by fluorescent *in situ* hybridisation (FISH). Y chromosome-positive epithelial cells were found in the lung, the skin and throughout the gastrointestinal tract, including the oesophagus, stomach, small intestine and colon. A highly purified population of MSCs, termed MAPCs (section 1.12.4), were

isolated from ROSA26 mice which constitutively express β-galactosidase. Following 55 – 65 population doublings, 1 or 10 – 12 MAPCs were microinjected into 3.5-day-old mouse blastocysts, which were then transferred to foster mothers and allowed to develop and be born. Chimeric animals were killed at 6 - 20 weeks, and BM-derived epithelial cells were observed in many somatic tissues, including the small intestine (Jiang et al., 2002). In biopsies from female patients who had undergone sex-mismatched HSC transplantation, *in situ* hybridisation for a Y chromosome-specific probe combined with immunohistochemical staining for cytokeratins, demonstrated mucosal cells of donor origin in the gastric cardia (Korbling et al., 2002). Similarly, in gastrointestinal biopsies from four long-term BM transplant survivors who had developed GvHD or intestinal ulceration, epithelial cells of BM origin were detected up to 8 years post-transplantation in the oesophagus, stomach, small intestine and colon (Okamoto et al., 2002), and a further recent paper describes the presence of donor-derived epithelial cells in colonic biopsies from 8 patients that received a sex-mismatched BM transplant (Spyridonidis et al., 2004). This study excluded the possibility that BM-derived intestinal epithelial cells are merely intraepithelial lymphocytes in close proximity with epithelial cells, by using a rigorous three dimensional confocal detection technique on tissue sections that were triple stained for donor-specific, epithelial-specific and haematopoietic-specific markers (Spyridonidis et al., 2004). It is important to note that donor-derived intestinal epithelial cells are single cells randomly interspersed throughout the crypts and villi, and the incidence of BM-derived intestinal epithelial cells post-transplant are variable and are generally low, ranging from 0.04% (Spyridonidis et al., 2004) to approximately 10% (Jiang et al., 2002). However, BM contribution to epithelial cells in human intestinal biopsies was increased between 5 – 50-fold in tissue damaged by GvHD, compared to intact epithelium (Okamoto et al., 2002; Spyridonidis et al., 2004), although the low levels of engraftment and the lack of donor-derived cell clusters in crypts or villi indicate that it is unlikely that the BM cells form intestinal epithelial stem cells or undergo proliferation.

BM cells also contribute to mesenchymal lineages in the mouse and human gastrointestinal tract. Transplanted BM cells contribute to large proportions of myofibroblasts and fibroblasts in the mouse lung, stomach, oesophagus, skin, kidney and adrenal glands. Indeed, following lethal irradiation and whole BM transplant, up to 64% of ISEMFs in the mouse stomach were BM-derived 6 weeks post-transplant (Direkze et al., 2003). Analysis of liver biopsies from patients with fibrosis showed that a significant proportion of myofibroblasts were BM-derived, and BM can maintain differentiated cell production within a damaged tissue for extensive lengths of time e.g., in one patient, approximately 23% of myofibroblasts in the fibrotic liver were BM-derived 2 years post-transplant (Forbes et al., 2004).

1.13.2) BM contribution to vascular lineages

As discussed in section 1.12.5, it is well documented that the BM contains progenitor cells that can differentiate to form both endothelial cells and smooth muscle mural cells of blood vessels, particularly in situations where vascular remodelling and regeneration is required (see Table 1.1). A recent paper showed that transplanted BM forms endothelial cells in the human skin, stomach and small intestine, which were observed up to 7 years post transplant (Jiang et al., 2004). Levels of BM engraftment were low, with BM contributing to only 2.3% and 1.7% of endothelial cells in blood vessels of the skin and gut, respectively (Jiang et al., 2004).

1.13.3) BM contribution to cells in the adult epidermis

Similar to the situation in the gastrointestinal tract, there are several reports of a BM origin of several differentiated adult lineages in the mouse and human epidermis. Following transplantation of a single purified HSC, cytokeratin-positive cells of donor derivation were observed in the mouse epidermis, including the postulated stem cell zone, although these cells did not appear to undergo clonal expansion and are therefore

unlikely to be epidermal stem cells (Krause et al., 2001). The contribution of transplanted BM cells to differentiated keratinocyte lineages is significantly increased in the regenerating mouse epidermis (Badiavas et al., 2003; Borue et al., 2004), supporting the notion that non-haematopoietic cell production by BM is enhanced in response to signals from damaged or diseased tissues. BM-derived epidermal cells are also seen in human skin, as female recipients of a male HSC transplant showed BM-derived epidermal cells as late as 354 days post-transplantation (Korbling et al., 2002). In a recent "skin reconstitution assay", adult BM cells were transplanted directly onto the wounded mouse epidermis and within 3 weeks epidermal keratinocytes, follicular epithelial cells, sebaceous gland cells, dendritic cells and endothelial cells of BM origin were observed (Kataoka et al., 2003).

Table 1.2) The contribution of BM to adult lineages in non-haematopoietic tissues.

Recipient Organ(s)	Donor Cell(s)	BM-derived cells	Fusion/ no fusion	Reference
Liver (rat)	BM	Oval cells Hepatocytes	Not investigated	(Petersen et al., 1999)
Liver (human)	BM	Myofibroblasts	Not investigated	(Forbes et al., 2004)
Liver (human)	BM	Hepatocytes	Not investigated	(Alison et al., 2000; Theise et al., 2000)
Liver (mouse)	HSC	Hepatocytes	Not investigated	(Lagasse et al., 2000)
Liver (mouse, human)	BM	Endothelium	Not investigated	(Gao et al., 2001)
Liver (mouse)	HSC	Hepatocytes	Fusion	(Camargo et al., 2004; Wang et al., 2003; Willenbring et al., 2004)
Liver, gut, skin (human)	HSC (peripheral blood)	Epithelial cells	No fusion	(Korbling et al., 2002)
Kidney (mouse, human)	BM	Tubular epithelium, glomeruli	Not investigated	(Poulsom et al., 2001)
Kidney (mouse)	BM	Mesangial cells, endothelium	Not investigated	(Cornacchia et al., 2001)
Kidney (human)	BM	Endothelium	No fusion	(Lagaaij et al., 2001)
Heart (mouse)	BM	Cardiomyocytes	Not investigated	(Orlic et al., 2001b)
Heart (mouse)	SP cells	Cardiomyocytes, endothelium	Not investigated	(Jackson et al., 2001)

CNS, heart, liver (mouse)	BM	Purkinje cells, cardiomyocytes, hepatocytes	Fusion	(Alvarez-Dolado et al., 2003)
CNS (mouse)	BM	Purkinje cells	Fusion	(Weimann et al., 2003a; Weimann et al., 2003b)
CNS (mouse)	BM	Neurons	Not investigated	(Brazelton et al., 2000; Mezey et al., 2000)
CNS (mouse)	BM	Microglia	Not investigated	(Simard and Rivest, 2004)
CNS (mouse)	BM	Endothelium, neurons	Not investigated	(Hess et al., 2002)
CNS (mouse)	BM	Microglia and astrocytes	Not investigated	(Eglitis and Mezey, 1997)
CNS (human)	HSC	Neurons, astrocytes, microglia	No fusion	(Cogle et al., 2004)
Gastrointestinal tract (human)	BM	Epithelial cells	Not investigated	(Okamoto et al., 2002)
Colon (human)	HSC	Colonocytes	No fusion	(Spyridonidis et al., 2004)
Intestine (mouse and human)	BM	Myofibroblasts	Not investigated	(Brittan et al., 2002)
Skin (mouse)	BM	Keratinocytes	No fusion	(Brittan et al., 2004)
Skin (mouse)	BM	Keratinocytes	No fusion	(Borue et al., 2004)
Cheek epithelium (human)	BM	Buccal cells	No fusion	(Tran et al., 2003)
Liver, lung, gut (mouse)	MAPC	Epithelial cells	Not investigated	(Jiang et al., 2002)
Liver, lung, gut, skin (mouse)	Single HSC	Epithelial cells	Not investigated	(Krause et al., 2001)

Lung, stomach, oesophagus, skin, kidney, adrenal (mouse)	BM	Myofibroblasts	Not investigated	(Direkze et al., 2003)
Liver, lung, skin (mouse)	BM	Epithelial cells	No fusion	(Harris et al., 2004)
Pancreatic tumour (mouse)	BM	Myofibroblasts, fibroblasts	Not investigated	(Direkze et al., 2004)

1.14) Stem cell plasticity: *de novo* cell generation or heterokaryon formation

Before any clinical application can be considered, it is vital to understand the mechanisms that regulate stem cell plasticity, and the processes that occur within a stem cell in order for it to assume a new, differentiated phenotype. It is becoming increasingly apparent that two separate mechanisms of stem cell plasticity exist; *de novo* cell generation through BM cell differentiation, and fusion of BM cells with pre-existing cells to form a cell with multiple, genetically-variant nuclei, a heterokaryon. The first evidence for cell fusion was shown in co-cultures of embryonic stem (ES) cells, with either neural stem cells (Ying et al., 2002), or BM stem cells (Terada et al., 2002), wherein the adult stem cells appeared to fuse with the 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 BM stem cells (Terada et al., 2002)).

The classic "gold standard" proof of principle model for adult stem cell plasticity was demonstrated by the rescue of the $Fah^{-/-}$ mouse from its fatal metabolic disease by BM stem cell transplantation (Lagasse et al., 2000) (section 1.4), which definitively showed that BM-derived hepatocytes were functionally normal. However, cytogenetic analyses showed that almost all the newly formed, Fah-synthesising hepatocytes in these transplanted mice expressed a karyotype indicative that fusion had occurred between a transplanted BM cell and a host hepatocyte (Vassilopoulos et al., 2003; Wang et al., 2003). It is important to consider that hepatocytes are frequently polyploid and express multiple sets of chromosomes, and it is thereby possible that the liver presents a unique

environment for the formation of fused hybrid cells (reviewed in (Alison et al., 2004)).

Transplanted BM stem cells have been shown to fuse with Purkinje cells in brains of both mice (Weimann et al., 2003b), and humans (Weimann et al., 2003a). In the SOD1 mouse, which develops a lethal form of human ALS, BM cells can form adult cells in the CNS, heart and skeletal muscle, thereby prolonging animals' survival (Corti et al., 2004) (section 1.13). Examination of nuclear content by FISH analyses suggested that BM-derived neurons may be a result of cell fusion, although in the brains of some male mice that had received a female BM transplant, neurons were seen that did not express a Y chromosome, which could be explained by *de novo* cell formation by transplanted female BM cells, or alternatively may simply be a result of incomplete detection by the FISH technique (Corti et al., 2004). Conversely, in the human brain, transplanted BM cells appear capable of neurogenesis and contribute to approximately 1% of all neurons for up to 6 years post-transplant. Analyses of autopsy brain specimens from 3 female patients that received a male BM transplant showed no evidence of fusion, as only one X chromosome was present in all male-derived neurons (Cogle et al., 2004).

Whilst studies of sex chromosomal content may prove ambiguous due to artefacts created by the *in situ* hybridisation technique, the Cre/lox recombinase system provides an elegant means of studying the mechanism of adult stem cell plasticity, using mice of the Z/EG Cre-reporter strain. The Z/EG transgenic mouse expresses a transgene cassette consisting of the chicken β-actin promoter driving expression of a β-galactosidase/neomycin-STOP reporter gene. This is flanked by two loxP sites and followed downstream by enhanced green fluorescent protein (eGFP) expression. Expression of β-galactosidase alone is driven by the β-actin promoter until Cre mediated recombination occurs, at which point the β-galactosidase-STOP DNA is excised and the downstream eGFP is expressed instead. BM from male Z/EG mice was

transplanted into female mice that ubiquitously expressed Cre, and cells within multiple tissues were analysed for eGFP or β-galactosidase expression i.e., any cell resulting from the fusion of a BM cell with a host cell should express eGFP, and any cell which expresses β-galactosidase is the product of BM cell differentiation. However, recent reports using the Cre/lox recombinase system have also proved inconsistent, as BM-derived epithelial cells in the mouse liver, lung and skin were shown by this method to form without cell fusion (Harris et al., 2004), whereas results of Alvarez-Dolado and colleagues (Alvarez-Dolado et al., 2003) using a similar system, clearly demonstrated that BM cells spontaneously fuse with hepatocytes in the liver, Purkinje cells in the CNS and cardiomyocytes in the heart, with no evidence of *de novo* generation.

Evidence of heterokaryon formation following stem cell transplantation in the gastrointestinal tract or skin has not been reported to date, and gastrointestinal epithelial cells derived from transplanted mobilised peripheral blood stem cells were reported to contain a normal ratio of sex chromosomes (Korbling et al., 2002). Furthermore, keratinocytes of BM origin were frequently observed in the mouse epidermis, which were shown to form with no evidence of cell fusion (Borue et al., 2004). An important observation was made in that in some reported incidences of spontaneous cell fusion, the original stem cell markers are repressed and the transplanted cells appear to reprogram their new "host" nuclei to produce a sustainable, functional cell (Weimann et al., 2003b). Additionally, the incidence of heterokaryons was shown to increase as a tissue ages, suggesting that BM-derived cells can divide (Weimann et al., 2003b). The underlying principle, that transplanted BM cells have the capacity to rescue an otherwise fatal metabolic disease, is maintained and should not be overlooked, although it is irrefutable that before we overlook, or indeed overstate, the capacity of adult stem cells to regenerate damaged or diseased tissue, further clarification of the mechanisms involved in this apparent stem cell plasticity is essential.

CHAPTER II

Materials and Methods

2.1) BM transplant

In order to investigate the contribution of BM to adult lineages in the gastrointestinal tract and skin, BM transplants were performed where the donor cells and their differentiated progeny could be identified by the presence of a specific chromosome or protein that was absent in the recipient tissues. The most commonly used marker of the transplanted BM marker was the Y chromosome, following a gender-mismatched BM transplant. Additionally, BM from transgenic mice that express the Green Fluorescent Protein (GFP) was transplanted into wild type mice, thereby providing an alternative method of detection of the donor-derived cells.

All animal procedures were carried out under UK Home Office procedural and ethical guidelines (Home Office Project Licence No. 70/5798). All mice were maintained in the Biological Resource Unit, Cancer Research UK, London Research Institute.

Six week old female recipient mice had their BM ablated by 12 Gray whole body irradiation in a divided dose, 3 hours apart, using an IBL 637 gamma irradiator equipped with a caesium 137 source (Cis-Bio International). This was immediately followed by a tail vein injection of approximately 1 x 10^6 whole BM cells from age-matched male mice. BM was flushed from both tibias and femurs of donor mice with sterile PBS using a 5 ml syringe, and cells were centrifuged at 1500 rpm for 2 x 5 minutes, supernatant discarded, and cells resuspended in 0.2 ml sterile

PBS. The number of nucleated cells was calculated using a haemocytometer, by the dye exclusion test using 0.01% Trypan blue. Cells were stored at 4°C for no more than 2 hours before transplant. Following transplant, the mice were housed in sterile conditions and specific protocols were performed in accordance each defined study (section 2.2). All mice were killed by CO_2 inhalation and exsanguination.

2.2) Outline of individual experimental studies

2.2.1) BM contribution to intestinal lineages

2.2.1.1) Murine study

Donor mice were male C57/ Black 6 (n = 10), and recipient mice were female C57/ Black 6 (n = 30). Groups of ten mice were killed at 7 days, 14 days and 6 weeks after BM transplant, colons and small intestines were harvested and fixed in NBF for 24 hours at room temperature. Colons and small intestines from male and female wild type mice, to be used as controls, were harvested and fixed in for 24 hours in NBF at room temperature.

2.2.1.2) Human study

Tissue from three female patients who had received unprocessed BM transplants from male donors was analysed. These patients had subsequently had one or more gastrointestinal biopsies for suspected graft versus host disease (Table 2.1). Biopsies were formalin fixed, routinely processed and paraffin wax embedded. All tissue, including male and female control intestinal tissues were obtained from the archives of Hammersmith Hospital, London, and all procedures were in accordance with local ethical guidelines.

Table 2.1) Details of patients' used in this study. All patients were female, and received an unprocessed BM transplant from a male donor.

Patient (age at biopsy)	Region of biopsy	Time elapsed between BM transplant and biopsy
Patient 1 (39)	Gastric antrum	3 months
Patient 2 (37)	Duodenum (D2)	26 months
Patient 3 (23)	Rectum	38 days

2.2.2) BM contribution to experimental colitis

Following reports that the contribution of BM to functional differentiated cells in non-haematopoietic tissues is heightened in damaged or diseased states, we used a well-established model of experimental colitis with similarities to Crohn's disease, to investigate this claim in the mouse colon.

Donor mice were male Balb/C (n = 18), and recipient mice were female Balb/C (n = 50). Six weeks after BM transplantation, animals were sedated by a 0.2 ml intraperitoneal injection of Hypnorm (fentanyl citrate 0.315 mg/ml plus fluanisone 10 mg/ml; Janssen Animal Health), diluted 1 in 10 in injectable water. TNBS (2 mg in 50% ethanol; Sigma) was administered intrarectally, with control groups given 50% ethanol only. Animals' body weights were recorded daily, and five animals from both the TNBS-treated group and the ethanol-treated control group were killed on days 1, 4, 6, 8 and 14. Colons were fixed in NBF for 24 hours at room temperature.

2.2.3) BM contribution to the normal and wounded epidermis

Donor mice were male TgN(GFPU)5Nagy (GFP; n = 20; Jackson Laboratories Strain #003115129) and recipient mice were female Sv/C57 Black/6 (n = 60). In the GFP mice, expression of GFP protein is driven by the chicken beta-actin promoter and CMV intermediate early enhancer, with ubiquitous transgene expression within all nucleated tissues (Hadjantonakis et al., 1998).

2.2.3.1) Spontaneous fusion

In the spontaneous fusion study, which aimed to investigate whether transplanted BM cells transform to produce non-haematopoietic lineages or whether they fuse with indigenous cells within a tissue to form a

heterokaryon, the same BM transplant procedure was applied using male GFP transgenic donors (n = 7) and male Sv/C57 Black/6 recipients (n = 20).

2.2.3.2) Cutaneous wounds

Six weeks post-transplantation, two full-thickness circular wounds, each 3 mm in diameter, were made to the mouse dorsal skin of 45 of the transplanted mice. Fifteen mice were killed at 4, 7 and 30 days post-wounding, and approximately 2 cm^2 sections of unwounded and wounded skin were fixed in NBF for 24 hours at room temperature. Mice were injected intraperitoneally with 5-bromo-2'-deoxyuridine (BrdUrd; 0.1 ml/10 g body weight of 10 mg/ml stock in distilled water; Sigma) 2 hours prior to killing, to label cells in S-phase.

2.2.3.3) Wholemount preparation from tail epidermis

The tail skin from mice killed at 4 and 7 days post-wounding, was split lengthways using a scalpel, peeled from the tail, and cut into approximately 5 mm^2 segments. Sections were incubated in 5 mM EDTA (CRUK) in PBS at 37°C for four hours, or until the epidermis could be peeled intact from the dermis. Epidermal sheets were fixed in 4% formal saline (Sigma) for 2 hours at room temperature, rinsed in PBS, and stored in PBS + 0.2% sodium azide (Sigma) in 24-well plates (Integra Biosciences Inc.) at 4°C prior to immunohistological analyses.

2.2.3.4) Harvesting and cultivation of mouse keratinocytes

Wild type Sv/C57 Black/6 mice (n = 8), GFP transgenic mice (n = 8), and the remaining 15 Sv/C57 Black/6 mice that were transplanted with GFP BM 6 weeks previously, were shaved and the dorsal skin was swabbed in 70% ethanol, washed in distilled water, and further rinsed in 2 x antibiotics solution (penicillin/streptomycin; Sigma) in PBS. 2 cm squares of skin were floated on 0.25% trypsin (Gibco BRL) for 16 hours at 4°C, or

until the epidermis could be peeled as an intact sheet from the dermis. Epidermal keratinocytes were resuspended in keratinocyte growth medium, filtered through a 70 μm cell strainer (BD Biosciences) to remove cornified sheets, and plated onto 35 mm tissue culture dishes pre-seeded with a mitomycin C-treated (4 μg/ml for 2-3 hours at 37°C; Sigma) J2 fibroblast feeder layer. Three weeks post-seeding, approximately 20 - 50 keratinocyte colonies were observed per dish, which were then stained by immunofluorescence (section 2.3.10).

2.3) Immunohistochemistry and *in situ* hybridisation

Immunohistochemistry for various antigens was used to establish cell phenotype, and in wild type mice transplanted with GFP BM, GFP protein could be identified immunohistochemically. In tissues of female mice that received a BM transplant from a male mouse donor, *in situ* hybridisation for the Y chromosome was used to distinguish the transplanted cells. Therefore, combined immunohistochemistry and *in situ* hybridisation permitted both lineage tracing and phenotypical analyses.

Information regarding primary and secondary antibodies is presented in Tables 2.2 and 2.3, respectively. For all immunohistochemical and *in situ* hybridisation protocols, appropriate positive and negative controls were used. Prior to routine use of any newly acquired antiserum, a serial dilution was tested on positive control sections of fixed tissue in order to establish the optimal dilution of each antibody. Normal sera and secondary antibodies were used at the highest concentration (i.e., lowest dilution factor) recommended by the manufacturer. To eliminate background staining, the primary antibody was further diluted, or if unsuccessful, a serial dilution series of the secondary antibody was performed.

2.3.1) Tissue embedding and sectioning

Fixed tissue was transferred to 70% ethanol and embedded in paraffin wax by the CRUK Histopathology Unit. Embedded tissue blocks were sectioned at either 4 μm or 10 μm using a Jung Biocut 2035 microtome, depending on whether the chosen method of image acquisition was light or confocal microscopy, respectively. Tissue sections were placed on frosted glass microscope slides (Thermo Shandon COLORFROST®) and dried overnight at 37°C. Sections were stored for up to one month at room temperature in sealed slide boxes.

2.3.2) Dewaxing and blocking of endogenous peroxidases - paraffin sections

Tissue sections were dewaxed by a 5 minute immersion in sulphur-free xylene (BDH) and rehydrated by 3 minute incubations in decreasing concentrations of ethanol (100%, 95%, 80%, 70% and 50%) in distilled water. Sections were then treated with 30% hydrogen peroxide (BDH) in methanol to block endogenous peroxidases, rinsed in tap water, and washed in PBS.

2.3.3) Immunohistochemical staining protocol - paraffin sections

With the exception of the anti-BrdUrd primary antibody (section 2.3.5), antigen retrieval was performed either by microwave treatment in preheated sodium citrate buffer for 10 minutes at 700 W, or by incubation in bovine trypsin (BDH) for 15 minutes at 37°C. Sections were then rinsed in tap water, washed in PBS, and pre-incubated in normal serum (DAKO) from the donor species of the secondary antibody for 15 minutes, diluted at 1:25 in PBS. Without rinsing, all sections were then incubated in primary antibody, followed by incubation in a species-specific biotinylated secondary antibody. A tertiary layer of either streptavidin-hydrogen peroxidase (strep-HRP) diluted to 1:500, or streptavidin-alkaline phosphatase (strep-AP) diluted to 1:50, was finally applied. Primary, secondary and tertiary layers were all diluted in PBS and incubated for approximately 45 minutes at room temperature. Tissue sections were washed for 3 x 5 minutes in PBS between each antibody layer, and following the tertiary layer.

Strep-HRP was detected by DAB (Sigma) for 2 – 10 minutes, until the brown colour of the DAB could be detected macroscopically. Sections were then washed in PBS for 2 x 5 minutes, rinsed in tap water and counterstained lightly with haematoxylin and eosin (section 2.3.4).

Strep-AP was detected using the Vector Red Alkaline Phosphatase Substrate Kit 1 (Vector Laboratories), prepared according to the manufacturers' instructions and applied to the tissue for 10 – 30 minutes, or until a red colour could be detected macroscopically. Sections were then washed in PBS prior to the *in situ* hybridisation protocol (section 2.3.8).

2.3.4) H & E Counterstain

A light H & E counterstain was applied to tissue sections by immersion in Coles' haematoxylin (Pioneer Research Chemicals) for 10 minutes, followed by a thorough rinse in flowing tap water and immersion in acid alcohol (1% v/v concentrated HCl in 70% ethanol) for approximately 10 seconds. Following a second rinse in tap water, sections were immersed in Scott's tap water (30 mM $NaHCO_3$, 160 mM $MgSO_4$) for 10 seconds, and were rinsed in tap water for a final time. Sections were then rapidly dehydrated through graded alcohols to xylene (BDH), and mounted in DPX (Sigma).

2.3.5) Acid hydrolysis pretreatment to detect BrdUrd

BrdUrd is a thymidine analogue that is incorporated into newly replicated DNA during the cell cycle. Therefore, the proportion of cells that incorporate BrdUrd is a measure of the percentage of cells that are in S-phase at the time of BrdUrd administration.

Tissue sections to be stained for BrdUrd immunoreactivity required acid hydrolysis prior to application of the primary antibody to remove histone proteins and partially denature the DNA, thereby rendering the incorporated BrdUrd accessible to the primary antibody. Sections were dewaxed and endogenous peroxidases were blocked (section 2.3.2), followed by an incubation in 2 M HCl for 5 minutes at 37°C. Sections were then rinsed well in tap water prior to immunohistochemical staining.

2.3.6) Immunofluorescence staining protocol - paraffin sections

2.3.6.1) Double-labelling protocol

For co-localisation experiments using a double immunofluorescence method, tissue was subject to antigen retrieval and incubation in normal serum, as above, and was then stained with two primary antibodies derived from distinct species. The primary antibodies were subsequently probed with species-specific secondary antibodies conjugated to different fluorophores. The antibodies were applied in the following sequence: first primary; first secondary; second primary; second secondary. All antibodies were diluted in PBS and incubated for 1 hour at room temperature, with 3 x 5 minute PBS washes between each antibody layer.

For co-localisation experiments wherein both primary antibodies of interest were raised in the same species e.g. rabbit anti-GFP and rabbit anti-K14 antibodies, an identical protocol was followed to that above, with the exception that the second primary antibody was directly conjugated to a monofunctional fluorescein dye (section 2.3.7), thereby eliminating the necessity for a secondary antibody layer to detect this antibody and preventing cross-reaction of secondary antibodies derived from the same species.

2.3.6.2) Triple-labelling protocol

The use of a primary antibody conjugated to a monofunctional fluorescein dye permitted triple-labelling of tissue sections. For this, the double labelling protocol for primary antibodies from two distinct species was followed as above. This was proceeded by incubation in a third primary antibody conjugated to a fluorescein dye (section 2.3.7), which fluoresced at a separate wavelength than the two secondary antibodies already applied to the tissue. Our first primary antibody was rabbit anti-GFP,

which was detected by the AlexaFluor®594 goat anti-rabbit IgG, and the second primary antibody was mouse anti-BrdUrd, which was detected by the AlexaFluor®488 goat anti-mouse IgG. The third primary antibody was anti-K14 which was directly conjugated to a Cy5 dye (section 2.3.7). All antibodies were diluted in PBS and incubated for 1 hour at room temperature, with 3 x 5 minute PBS washes between each antibody layer.

Following immunofluorescence staining, tissue sections were either mounted in Citifluor (Citifluor Ltd.) and observed using a Zeiss LSM 510 confocal microscope (section 2.4), or were rinsed in PBS prior to the *in situ* hybridisation protocol (section 2.3.8).

2.3.7) Labelling of anti-K14 antibody with a monofunctional fluorescent dye

The mouse anti-K14 antibody was concentrated to 10 μM and the buffer was exchanged for 100 mM bicine (pH 8.5; Sigma) using a Centricon-30™ centrifugal concentrating device (Millipore). Monofunctional Cy3™ dye (Amersham International) was dissolved in dry N,N-dimethylformamide (Sigma) at a concentration of 10 mM. To initiate protein labelling, a 30-fold molar excess of dye relative to protein was added to 100 μg of the antibody. After 30 minutes at room temperature with occasional mixing, labelled antibody was separated from unconjugated dye by using a 6 kDa molecular weight cut-off disposable chromatography column (Biorad) equilibrated with PBS.

2.3.8) Combined immunohistochemistry and *in situ* hybridisation

2.3.8.1) Mouse tissue

Following immunohistochemistry, tissue sections were analysed by *in situ* hybridisation for detection of the Y chromosome. Sections were

incubated in 1M sodium thiocyanate (NaSCN; Sigma) in distilled water for 10 minutes at 80°C, washed in PBS, and digested in 0.4% w/v pepsin (Sigma) in 0.1M HCl at 37°C for varying times between 30 seconds and 25 minutes. The protease was quenched in 0.2% glycine (Merck and Co. Ltd.) in double concentration PBS, the sections were rinsed in PBS, post-fixed in 4% paraformaldehyde for 2 minutes, dehydrated through graded alcohols and air dried. A FITC-labelled Y chromosome paint (Star-FISH, Cambio) in the supplier's hybridisation mix was added to the sections, sealed under a glass coverslip, heated to 60°C for 10 minutes, and incubated overnight at 37°C. Slides were washed in 50% formamide / 2 x SSC (3 x 5 minutes), 2 x SSC (3 x 5 minutes), and 4 x SSC / 0.05% Tween-20 (Q-Biogene) for 10 minutes, all at 37°C. Slides were washed with PBS and sections to be viewed by direct fluorescence were mounted in Citifluor (Citifluor Ltd.) and observed using a Zeiss LSM 510 confocal microscope (section 2.4). Tissue sections that were to be viewed indirectly by a light microscope, were incubated with peroxidase-conjugated anti-fluorescein antibody (Boehringer Mannheim) diluted to 1: 250 in PBS, for 60 minutes. Slides were developed in DAB for 1 – 2 minutes, counterstained lightly with haematoxylin and eosin (section 2.3.4), and mounted in DPX.

2.3.8.2) Human tissue

For Y chromosome detection in human tissues, an identical protocol to mouse tissue was used, with a FITC-labelled human Y chromosome paint (Star-FISH, Cambio). Tissue sections were heated to 80°C for 10 minutes to hybridise the probe, and then incubated overnight at 37°C.

2.3.9) Immunofluorescence staining protocol: epidermal wholemounts

Protocol was performed in 24-well plates (Integra Biosciences Inc.). All antibodies were diluted in PB buffer, and incubated overnight with gentle agitation. Tissue was washed for 4 hours in PBS + 0.2% Tween-20 (Q-Biogene) between each antibody layer. Epidermal wholemounts were blocked and permeabilised by incubation in PB buffer for 30 minutes before immunofluorescence double-labelling. Anti-GFP primary antibody was applied to tissues, and was detected by the AlexaFluor®593 goat anti-rabbit IgG. Anti-K14 antibody directly conjugated to a monofunctional Cy5 dye (section 2.3.7) was then applied to the wholemounts, prior to rinsing in distilled water and mounting in Gelvatol (Monsanto Chemicals) containing 0.5% 1, 4-Diazabicyclo[2.2.2]octane (DABCO; Sigma). Wholemounts were then observed using a Zeiss LSM 510 confocal microscope (section 2.4).

2.3.10) Immunofluorescence staining protocol: keratinocyte colonies

Approximately 3 weeks after harvesting and cultivation of keratinocyte colonies from wild type (Sv/C57 Black/6) mice, GFP transgenic mice, and wild type mice transplanted with GFP BM 6 weeks previously (section 2.2.3), a double-labelling immunofluorescence method was performed. The culture medium was discarded from each 35 mm plate and cells were rinsed in PBS and fixed in 4% formaldehyde for 20 minutes at room temperature. Cells were then permeabilised in 0.1% Triton X-100 (Sigma) in PBS for 4 minutes, and washed in PBS before immunostaining. Cultures were incubated in anti-GFP primary antibody, which was detected by a biotin-conjugated goat anti-rabbit IgG (Biosource). A streptavidin-fluorescein conjugate (Biosource) was applied as the tertiary layer at 1: 100, and cells were finally incubated in an anti-K14 antibody conjugated to a monofunctional Cy3 dye (section

2.3.7). All antibodies were diluted in PBS and applied to the cultures for 2 hours. Cultures were washed 2 x 5 minutes in PBS between each antibody layer. Finally cultures were mounted in Citifluor (Citifluor Ltd.) and images were captured using a Hamamatsu digital camera (Improvision) on a Zeiss Axioplan fluorescence microscope (Carl Zeiss Ltd.).

2.4) Confocal Imaging

Fluorescent images of both wholemount tissue and immunofluorescence-stained paraffin sections were acquired with a Zeiss LSM 510 confocal microscope (Carl Zeiss Ltd.), using two argon lasers at 488 nm and 568 nm and a helium-neon laser at 633 nm.

2.4.1) Wholemount tissue

In the wholemount tissue, epidermal sheets were scanned from the dermis to the epidermal surface, encompassing the hair follicle bulb to the basal interfollicular epidermis, to an approximate thickness of 40 – 80 μm. Approximately 30 optical sections of each sheet were captured with a typical increment of 1 – 3 μm, and scans were presented as z-projections.

2.4.2) Paraffin sections

Paraffin sections on glass microscope slides were scanned widthways to an approximate thickness of 10 μm. Approximately 20 optical sections were captured with a typical increment of 0.5 μm, and scans were presented as z-projections.

2.5) Polymerase chain reaction (PCR)

We performed PCR on DNA isolated from keratinocyte colonies from female wild type mice transplanted with male GFP BM that were immunoreactive for both GFP and K14 (section 2.3.10). Therefore, the identification of both GFP- and Y chromosome-specific sequences in DNA isolated from the keratinocyte colonies would supplement the histological data. All reagents used were of molecular biology grade.

2.5.1) Isolation of colonies for DNA extraction

Keratinocyte colonies from female wild type mice transplanted with BM from GFP male transgenic mice, that were immunoreactive for both GFP and K14 antigens (section 2.3.10), were "picked" using a sterile needle and transferred to sterile T75 cell culture flasks containing keratinocyte growth medium. Positive and negative DNA control colonies were "picked" in the same way from GFP transgenic mice and from wild type mice that did not receive a BM transplant, respectively.

2.5.2) DNA extraction protocol

For the extraction of DNA from keratinocyte colonies, the following protocol was performed. Proteinase K (stock 20 mg/ml in distilled water; Sigma) was added to digest buffer to give a final concentration of 100 μg/ml. Keratinocyte growth medium was removed from the T75 cell culture flasks containing the colonies, and replaced with 750 μl of the proteinase K solution. Flasks were incubated overnight at 56°C, and non-adherent cells were scraped into the buffer and transferred to a 2 ml sterile eppendorf. Isopropanol (750 μl; Sigma) was added to precipitate the DNA and the tubes were vortexed briefly, and then centrifuged at 2600 rpm for 20 – 30 minutes. Most of the supernatant was discarded, except a small volume containing the DNA sample, which was washed

with 70% ethanol and dried overnight at 37°C. TE buffer (1.5 ml) was then added to dissolve DNA, and DNA was stored at -20°C, indefinitely.

2.5.3) PCR

The CRUK Oligonucleotide Service created all primers used in PCR. PCR for the GFP transgene was carried out using the following oligonucleotide primers to produce a 306-bp product:

GFP-1f (5'-TGA ACC GCA TCG AGC TGA AGG G-3'
GFP-2r (5'-TCC AGC AGG ACC ATG TGAT CGC-3'

DNA loading was assessed using the following wild type primers:

B2-1 (5'-CAC CGG AGA ATG GGA AGC CGA A- 3'
B2-2 (5'-TCC ACA CAG ATG GAG CGT CCA G-3'

For each reaction, 1 μl of DNA was mixed with 0.5 μl of each primer; GFP-1f, GFP-2r, B2-1 and B2-2, 2 μl dNTP (Takara Bio), 2.5 μl 10X buffer (Takara Bio), 0.25 μl Takara Taq (Takara Bio), and 17.75 μl distilled water. PCR reaction conditions were as follows: denaturation at 96°C, 20 s; extension at 55°C, 20 s; and annealing at 72°C, 45 s. Thirty five amplification cycles were performed.

2.5.4) PCR using the incorporation of a radioactive label

The presence of the Sex Determining Region (SDR) of the Y chromosome was identified by radioactive PCR. The primers used to produce an 185-bp product were as follows:

5'- GAC TAG ACA TGT CTT AAC ATC TGT CC-3'
5'- CCT ATT GCA TGG ACA GCA GCT TAT G-3'

The reaction mix was supplemented with nucleic acids labelled with radioactive phosphorous ([α^{32}P]dCTP; Amersham International) to label the internal primers. For each reaction, 1 μl of DNA was mixed with 1 μl of each primer, 2 μl dNTP (Takara Bio), 2 μl [α^{32}P]d-CTP, 2.5 μl 10X buffer (Takara Bio), 0.25 μl Takara Taq (Takara Bio), and 15.75 μl distilled water. The PCR reaction conditions were as follows: denaturation at 94°C, 60 s; extension at 58°C, 60 s; and annealing at 72°C, 60 s. Thirty amplification cycles were performed. Products were resolved on a 1.8% agarose gel (section 2.5.5), which was then rinsed briefly in tap water and then soaked in 20 x SSC for 20 minutes. The DNA was transferred from the gel to a Zeta probe membrane (Biorad) by capillary transfer overnight. This was followed by cross-linking the membrane with UV light to immobilise the DNA to the membrane. The membrane was then exposed to Kodak film at -80°C for 4 hours.

2.5.5) Electrophoresis

PCR products were separated on a 1.8% agarose gel. A 300 ml agarose gel was made by mixing 2.4 g ultra pure agarose (Gibco BRL) with 150 ml distilled water, microwaving at high power for 3 – 5 minutes until the agarose was dissolved, and then adding 120 ml distilled water, 30 ml TE buffer and 5 μl of ethidium bromide (Sigma). The whole solution was poured in a large gel tank (Scotlab-Anachem Ltd.), whose sides were sealed with autoclave tape to generate a closed chamber, and 2 x 28-well combs were immersed in the agarose to create wells. The solution was left to set into a gel for approximately 30 minutes. The combs were removed, and 25 μl of amplified PCR products were then mixed with 5 μl DNA loading buffer, loaded into the gel, and separated at 150 V for 1 – 2 hours. 1kb and 100bp DNA ladders (NEB) were run alongside to determine fragment size. DNA bands were visualised under UV light and photographed using an EagleEye Still Video System (Stratagene).

2.6) Statistical analyses

Data were analysed using GraphPad Prism version 4.0 (GraphPad software), using either Mann Whitney tests (two-tailed T-tests) or one-tailed T-tests, with statistical significance defined as $P < 0.05$.

Table 2.2) Details of primary antibodies used throughout the project.

Antibody	Specificity	Species	Dilution in PBS	Antigen Retrieval	Source
SMA	Smooth muscle actin	Mouse	1:4000	N/A	Sigma
Desmin	Desmin	Mouse	1: 20	Microwave	Euro-Diagnostica
F4/80	Macrophages	Rat	1: 50	Trypsin	Serotec
CD34	Haematopoietic progenitors, Keratinocyte progenitors	Rat	1: 100	Microwave	Cedarlane Labs. Ltd.
ICAM-1	Endothelial cells	Rat	1: 20	Microwave	CRUK
GFP	Green fluorescent protein	Rabbit	1: 1000	Microwave	Molecular Probes
K14	Basal keratinocytes	Rabbit	1: 10,000	Microwave	BAbCO
Ly6G	Neutrophils	Rat	1: 100	Microwave	BD Biosciences
BrdUrd	Cells in S-phase	Mouse	1: 25	Acid hydrolysis	BD Biosciences

Table 2.3) Details of secondary antibodies used throughout the project.

Immunoglobulin	Dilution	Source
Rabbit anti-mouse IgG biotin conjugate	1: 250	DAKO
Rat anti-mouse IgG biotin conjugate	1: 100	Vector Labs.
Swine anti-rabbit biotin conjugate	1: 500	DAKO
Goat anti-rabbit biotin conjugate	1:200	Biosource International
Goat anti-rabbit IgG Cy™5 conjugate	1: 1000	ZyMax
Goat anti-mouse IgG Cy™5 conjugate	1: 100	ZyMax
AlexaFluor®594 goat anti-rabbit IgG	1: 100	Molecular Probes
AlexaFluor®488 goat anti-rat IgG	1: 100	Molecular Probes
AlexaFluor®488 goat anti-mouse IgG	1: 1000	Molecular Probes
AlexaFluor®647 goat anti-rat IgG	1: 100	Molecular Probes

BUFFERS AND SOLUTIONS

Unless otherwise stated, distilled water refers to 'Milli-Q Plus' water produced by the reverse osmosis 'ultra pure water system' (Millipore).

Tissue Fixation

Neutral buffered formalin (pH 7.0) (NBF, CRUK)

Formalin, full strength (37-40% formaldehyde)	100 ml
Na_2HPO_4 (BDH)	6.5 g
NaH_2PO_4 (BDH)	4 g
Distilled water	900 ml

4% Paraformaldehyde

Paraformaldehyde (Sigma)	16 g
PBS (CRUK)	400 ml

Paraformaldehyde dissolved in PBS at 70°C, cooled to room temperature before use.

Immunohistochemistry

Phosphate buffered saline (PBS, CRUK)

NaCl (Sigma)	8.0 g
KCl (BDH)	0.35 g
Na_2HPO_4 (BDH)	1.43 g
KH_2PO_4(BDH)	0.25 g
Distilled water	1 L

Adjusted to pH 7.2 and autoclaved.

TBS Buffer (pH 7.2)

NaCl (Sigma)	0.9%
Hepes (Sigma)	20 mM

PB Buffer

Skimmed milk powder	0.5%
Fish skin gelatin (Sigma)	0.25%
Triton-X-100 (Sigma) in TBS	0.05%

Bovine trypsin (pH 7.8)

Bovine pancreas trypsin (BDH)	100 mg
$CaCl_2$ (Sigma)	100 mg
Distilled water	100 ml

Heated to 37°C.

Diaminobenzidine (DAB)

DAB (Sigma)	0.05%
Hydrogen peroxide (BDH)	0.001%
PBS	9 ml

In situ hybridisation

10X salt solution

NaCl (Sigma)	176.2 g
Na_2HPO_4 (BDH)	14.2 g
1M Tris-HCl (Sigma)	100 ml
0.2M EDTA (Sigma)	250 ml

The *di*-sodiumhydrogenphosphate was dissolved in water and adjusted to pH 6.8. The sodium chloride, Tris-HCl and EDTA were then added in order, and the volume was made up to 1 L with water and stored at room temperature.

20 X SSC (pH 7.5) (CRUK)

NaCl (Sigma)	175.5 g
Na citrate (Sigma)	88 g

Distilled water to 1L total volume.

Formamide buffer

10X salt solution	250 ml
Formamide (non-deionised) (Sigma)	1.25 L

Distilled water to 2.5 L total volume.

Sodium citrate buffer (pH 6.0)

Tri-sodium citrate (Sigma)	2.94 g
Distilled water	1 L

Cell Culture

Keratinocyte growth medium (CRUK)

FAD powder (Imperial Labs.) consisting of 1 part of Ham's F12 medium and 3 parts Dulbecco's modified Eagle's medium (CRUK), plus 0.18 mM adenine (final concentration; Sigma), was supplemented with 100 IU/L penicillin (Gibco BRL) and 100 μg/L streptomycin (Gibco BRL). FAD medium (CRUK) was bubbled with CO_2 until acidic in pH, before sterilising by filtration through a 0.22 μm filter (BD Biosciences). Medium was stored at 4°C until use.

Stock solutions of additives were prepared. 10^{-5} cholera endotoxin (ICN) was stored at -20°C. 10 ng/ml epidermal growth factor (Gibco BRL) was prepared by first dissolving in 1:10 volume of 0.1 M acetic acid (BDH) before adding to FAD medium containing 10% fetal calf serum (FCS, Imperial Labs.) and stored at -20°C. The additives were combined into a 1000x cocktail (HICE): 1ml hydrocortisone, 100 μl cholera endotoxin and 1 ml epidermal growth factor stock solutions were added to 7.9 ml FAD medium with 10% FCS and stored at -20°C. The final concentrations in

the medium were 10^{-10} M cholera endotoxin, 0.5 g/ml hydrocortisone and 10 ng/ml epidermal growth factor. 1000x insulin stock solution (5 mg/ml in 5 mM HCl, Sigma) was stored at -20°C. The final concentration in the medium was 5 μg/ml insulin. Complete keratinocyte medium (FAD+FCS+HICE) was prepared by adding 10% (v/v) FCS cocktail and insulin solutions to the FAD medium prior to use, and stored at 4°C for up to 14 days.

Polymerase Chain Reaction

Tris/EDTA (TE) Buffer

1 M Tris (pH 7.5, Sigma)	1 ml
0.5 M EDTA (CRUK)	200 μl
Distilled water	98.8 ml

DNA gel loading buffer (6x)

Bromophenol blue (Sigma)	0.25%
Xylene cyano (Sigma)	0.25%
Ficcol (Sigma) in distilled water	15%

Digest buffer

1M Tris-HCl (pH 8.5, Sigma)	25 ml
0.5 M EDTA (pH 8, CRUK)	2.5 ml
5 M NaCl (Sigma)	10 ml
20% Sodium dodecyl sulphate (SDS)	2.5 ml
Distilled water	210 ml

SUPPLIERS AND DISTRIBUTORS

Amersham International	Buckinghamshire, UK.
BabCO	Richmond, CA, USA
BDH Laboratory Supplies Inc.,	Hertfordshire, UK.
BD Biosciences	New Jersey, USA
Bio-Rad Laboratories Inc.,	Hertfordshire, UK.
Biosource International	Camarillo, CA, USA
Boehringer Mannheim UK Ltd.,	East Sussex, UK.
Cambio Ltd.,	Cambridge, UK
Carl Zeiss Ltd.,	Hertfordshire UK.
Cedarlane Laboratories Ltd.,	Ontario, Canada
Cis-Bio International	Cedex, France
Citifluor Limited	London, UK
DAKO A/S	Cambridge, UK.
Euro-Diagnostica	Malmo, Sweden
Gibco BRL/Life Technologies Ltd.,	Renfrewshire, UK.
GraphPad Software	San Diego, CA, USA.
ICN Pharmaceuticals Ltd.,	Thame, Oxon, UK.
Imperial Laboratories (Europe Ltd)	Hampshire, UK.
Improvision	Coventry, UK.
Integra Biosciences Inc.	Fareham, Hants, UK.
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Merck and Co. Inc.	New Jersey, USA
Millipore	Watford, UK
Molecular Probes	Leiden, Netherlands
Monsanto Chemicals	Massachusetts, USA.
New England Biolabs (NEB)	New York, USA
Pioneer Research Chemicals	Colchester, UK
Q-Biogene	Carlsbad, CA, USA
Scotlab-Anachem Ltd.,	Strathclyde, UK
Serotec	Oxford, UK

Sigma Chemical Co.,	Dorset, UK
Stratagene	La Jolla, CA, USA
Takara Bio	Gennevilliers, France
Vector Laboratories	Burlingame, CA, USA
ZyMax	Cambridge, UK

CHAPTER III

BM cells contribute to ISEMFs in the mouse and human small intestine and colon

3.1) Introduction

3.1.1) BM contributes to gastrointestinal epithelial lineages

Several studies have shown that a population of progenitor cells exists in the BM and peripheral blood, which can give rise to cytokeratin-expressing epithelial lineages in the mouse and human gastrointestinal tract (Jiang et al., 2002; Korbling et al., 2002; Krause et al., 2001; Okamoto et al., 2002; Spyridonidis et al., 2004). However, the bulk of evidence indicates that BM is unlikely to have a therapeutic relevance in mucosal regeneration by the direct production of epithelial cells. Firstly, the reported incidence of gastrointestinal epithelial cells of BM origin is very low, ranging from 0.04% (Spyridonidis et al., 2004) to approximately 10% (Jiang et al., 2002). Secondly, the observed donor-derived gastrointestinal epithelial cells are present as single cells within the gastric glands and intestinal crypts and villi, and there are no reports of cell clusters, thus implying that BM-derived gastrointestinal epithelial cells do not behave as stem cells and proliferate to produce differentiated cell progeny.

The paradigm that BM enhances its contribution to cells in non-haematopoietic tissues in damaged or diseased states (section 1.13) appears to also apply for the gastrointestinal epithelium, as numbers of BM-derived epithelial cells were increased between 5 - 50-fold in patients who had received a sex-mismatched BM transplant and had

subsequently developed GvHD or intestinal ulceration (Okamoto et al., 2002; Spyridonidis et al., 2004). However, the observed fraction of BM-derived epithelial cells in the human colon in GvHD was still relatively minor at only 0.22% (Spyridonidis et al., 2004). In a separate study, less than 4% of enterocytes in the small intestine were of BM origin in patients with GvHD, which regressed to less than 1% 2 years post-transplant when patients had recovered (Okamoto et al., 2002), further evidence that BM does not form a sustainable epithelial stem cell population in the gastrointestinal tract.

3.1.2) The crypt microcolony assay: a possible explanation for the lack of BM-derived intestinal epithelial stem cells

The ability of stem cells to regenerate epithelial cell populations of entire intestinal crypts and villi following cytotoxic treatment has been widely studied using the crypt microcolony assay, first described by Withers and Elkind in 1970 (Withers and Elkind, 1970). In this assay, crypt survival levels, the growth rate of surviving crypts and the number of clonogenic cells in each crypt of the mouse intestine are measured following whole body gamma irradiation. Four days after irradiation, the surviving clonogenic cells in a crypt divide to form microcolonies and regenerate all of the epithelial cell populations of that crypt, and the overlying small intestinal villi. The newly repopulated crypts are 1.5 - 2-fold bigger than normal crypts, and they divide by crypt fission to regenerate the entire intestinal mucosa (reviewed in (Roberts et al., 2003)). Crypts that have had their stem cells depleted by irradiation are reproductively sterile and consequently undergo apoptosis and disappear, but are identified by the radioresistant Paneth cells that remain in the crypt base. The number of clonogenic cells within a crypt following irradiation is variable and appears to be age-dependent, as older mice have fewer surviving crypts and display a two-fold increase in the level of crypt cell apoptosis, although having a greater number of clonogenic cells (Martin et al., 1998a; Martin et al., 1998b). The dose of irradiation and degree of mucosal damage also appears to influence clonogenic cell number, with reports of between

5 and 10 clonogenic cells following low dose irradiation in the mouse colon, and up to 36 clonogenic cells per crypt in extremely damaged mucosa following high-dose irradiation in both the small intestine and colon of mice (Cai et al., 1997; Hendry et al., 1992). It is suggested that in severely damaged mucosa, stem cells recruit their daughter cells to the niche to increase clonogenic cell number (reviewed in (Roberts et al., 2003)). However, Wright and colleagues showed that single stem cells are frequently retained within a crypt following severe radiation damage, and these cells can reproduce the entire repertoire of adult lineages in the intestinal epithelium, thereby forming a monoclonal crypt (Wright and Alison, 1984).

The results of these early assays may provide an explanation for the apparent lack of BM-derived epithelial stem cells in intestinal crypts following irradiation and BM transplantation in the aforementioned stem cell plasticity studies (section 3.1.1). The capacity of the intestinal epithelial stem cell(s) to undergo multipotent differentiation and regenerate the entire intestinal mucosa demonstrates the highly efficient nature of these cells, and indicates that a reserve population of stem cells located in the BM is not essential. Moreover, BM-derived epithelial cells are reportedly interspersed randomly within the intestinal crypts and villi in both mice and humans, and it can therefore be assumed that these cells are short-lived due to the high cell turnover in these tissues. The functional relevance of BM-derived intestinal epithelial cells is as yet unclear. They do not appear to contribute to sustainable epithelial stem cell populations in the intestinal crypts, although it is possible that they assist in the repair of damaged mucosa by producing low numbers of epithelial cells.

A very recent study supports the likelihood that BM contribution to gastrointestinal lineages occurs relative to the level of tissue damage. Wild type mice were transplanted with whole BM from β-galactosidase-expressing ROSA26 mice and were then either given gastric ulcers, or were infected with *Helicobacter*, a known carcinogen. In transplanted

mice that received no further manipulation or developed acute gastric ulceration, no BM-derived epithelial cells were observed in the gastric glands. However, in mice that were infected with *Helicobacter*, less than 5% of epithelial cells in gastric glands were BM-derived 5 weeks after infection, which increased dramatically to approximately 90% 1 year after infection in high-grade gastrointestinal intraepithelial neoplasia. In addition, a proportion of these cells incorporated BrdUrd and were therefore capable of proliferation, the first evidence that BM-derived cells in the gastrointestinal tract can undergo DNA synthesis (Houghton et al., 2004).

Therefore, it is possible that the level of tissue regeneration induced by whole body irradiation, GvHD, or ulceration requires only a minimal contribution from cells in the BM, although in high-grade neoplasia it is likely that the endogenous intestinal stem cells are not sufficient to regenerate the damaged tissue and thus a supplementary stem cell population in the BM is employed.

3.2) Aim

The aim of this section of the project was to investigate the contribution of BM cells to differentiated adult lineages of the mouse and human gastrointestinal tract. The role of BM cells in the formation of gastrointestinal epithelial cells has previously been demonstrated, although this appears to be minimal, and long term engraftment and proliferation of BM-derived gastrointestinal cells has only been shown in a model of gastric neoplasia (Houghton et al., 2004). The critical importance of epithelial:mesenchymal cell paracrine crosstalk in the regulation of intestinal function is well documented, in particular the close interactions between the intestinal epithelial cells and the ISEMFs (reviewed in (Powell et al., 1999a; Powell et al., 1999b)). Little is known about the origin of ISEMFs and I therefore decided to investigate the contribution of BM to mesenchymal cell types in the small intestine and colon of mice and humans. I now present further verification of the plasticity of adult BM cells, by showing that they provide a major contribution to the ISEMFs in the small intestine and colons of both mice and humans.

3.3) Methods

Small intestines and colons were harvested from female mice that had received a lethal dose of irradiation and were immediately rescued by a whole BM transplant from male donors (section 2.1). Murine tissues were harvested at 1, 2 and 6 weeks post-transplant. Gastrointestinal biopsies were obtained from female patients that had developed GvHD following unprocessed BM transplant from male donors (Table 2.1). In both the mouse and human studies, the donor BM cells were male and the recipient tissues were female, which presented the Y chromosome as a marker of the transplanted cells. The Y chromosome was readily detected in paraffin-embedded tissue sections by *in situ* hybridisation, and this was combined with immunohistochemistry for various antigens to determine the phenotype of the donor-derived cells (section 2.3). Male and female control intestinal tissue from humans and mice were used as positive and negative controls for the Y chromosome *in situ* hybridisation protocol.

A more detailed description of the materials and methods used in this chapter is given in section 2.2.1.

3.4) Results - murine studies

3.4.1) Male and female controls

As paraffin sections analysed by *in situ* hybridisation for Y chromosome-specific sequences were 4 μm thick, the Y chromosome was not present in all nuclear profiles and the observed proportion of donor-derived cells in female colons following a male BM transplant was therefore underestimated. *In situ* hybridisation for the Y chromosome in the colons of male control mice showed that approximately 69.2% of ISEMFs contained a Y chromosome, whereas there were no Y chromosome-positive cells in the female control colons (Figure 3.1). Accordingly, the observed values of donor cells in the female transplant tissues were divided by a factor of 0.692 to reflect total BM-derived cell populations.

3.4.2) BM-derived cells in the mouse intestine following BM transplant

Following transplantation of wild type male BM into irradiated wild type female mice, *in situ* hybridisation for Y chromosome expression showed frequent Y chromosome-containing cells within the small intestines and colons at all timepoints investigated (Figures 3.2 – 3.6). The vast majority of these cells were located within the lamina propria subjacent to the epithelial mucosa, and donor-derived cells were rarely seen within the intestinal crypts or villi.

3.4.3) Identification of ISEMFs derived from the BM

Several lines of evidence support the contention that many of the Y chromosome-containing cells in the female recipients were myofibroblast in nature. These cells were spindle-shaped and were located in the subepithelial lamina propria, often present as cellular columns spanning

from the base of the crypt to the intestinal lumen. Immunohistochemical analyses confirmed the ISEMF phenotype of the donor-derived cells, which were strongly SMA-positive and did not express desmin (Figure 3.7 A). Furthermore, some BM-derived cells were clearly negative for the haematopoietic progenitor marker CD34 (Figure 3.7B), and the mouse macrophage marker F4/80 (Figure 3.7C), which excluded the possibility that these cells were merely infiltrating inflammatory lineages.

3.4.4) A significant number of ISEMFs in the mouse are derived from the BM

Based upon morphological criteria and the co-expression of a nuclear Y chromosome and cytoplasmic SMA expression, I detected BM-derived cells in the lamina propria of the mouse small intestine and colon. As early as 7 days post-BM transplant there was evidence of a few donor-derived ISEMFs in the lamina propria (Figure 3.2). These cells appeared to be single entities, randomly interspersed throughout the lamina propria, and rows or clusters of BM-derived ISEMFs were not seen at this stage. In both the small intestine and colon, the numbers of BM-derived ISEMFs appeared to intensify with increasing time elapsed after transplant. Numbers of BM-derived ISEMFs were calculated in tissue sections from the mouse colon at 2 and 4 weeks post-transplant. At 2 weeks post-transplant, a vast increase in BM-derived ISEMFs was observed, and BM contributed to 49.5% of these cells at this stage (Table 3.1; Figure 3.3). This was further increased at 6 weeks post-transplant, as 57.6% of ISEMFs were of male origin in the mouse colon (Table 3.1; Figures 3.4, 3.5).

3.4.5) BM-derived ISEMFs form cellular columns, extending upward in the lamina propria

In the small intestine and colon at 2 and 6 weeks post-transplant, I frequently observed columns of BM-derived ISEMFs extending throughout the lamina propria from the pericryptal zone at the base of the

crypt, forming a network as they projected upwards until reaching the intestinal lumen (Figure 3.5). This suggests that BM-derived ISEMFs are functionally competent and are interconnected within a cellular network, which proliferates and migrates upward to the intestinal lumen. It is possible that the BM-derived ISEMFs derive from a progenitor cell near the base of the crypt, although the lack of markers of proliferation in these tissues prevented the irrefutable demonstration of the proliferative capacity of these cells.

3.4.6) BM-derived ISEMFs appear to contribute to the epithelial stem cell niche

The intestinal epithelial stem cells are housed at the base of the crypts within the stem cell niche, which is composed of the underlying mesenchymal cells and their ECM substratum. Due to the prominent expression of ISEMFs in the lower two-thirds of the crypts (Neal and Potten, 1981), and their stringent regulation of epithelial cell function, it is postulated that ISEMFs comprise a major component of the intestinal stem cell niche. I frequently observed BM-derived ISEMFs in the lamina propria adjacent to the epithelial cells in the base of the crypts, thus indicating that BM may be a major source of the cells of the intestinal stem cell niche (Figure 3.6).

3.5) Results - Human studies

In male control intestinal biopsy material, ISEMFs were positive for the Y chromosome. In the female intestinal control tissues, a Y chromosome was not detected within any cell nuclei (Figure 3.8).

In female patients who received a BM transplant from male donors, *in situ* hybridisation for the Y chromosome revealed that many cells of donor origin were present in the intestinal biopsy material. Some of these cells displayed positive immunoreactivity for SMA and thus, in addition to morphological criteria, were identified as ISEMFs (Figure 3.9). BM-derived ISEMFs were present in tissue biopsies from all patients examined, although the frequency of these cells varied between each patient. There was no distinct pattern of expression of these cells in the villous tips or crypt bases, and their expression appeared to be random throughout the lamina propria. In this small patient series, there was no obvious correlation between the degree of BM engraftment and the time elapsed between BM transplant and tissue biopsy.

3.6) Discussion

This study verifies recent claims that cells in the adult BM exhibit a remarkable plasticity. I demonstrate that an organised network of myofibroblasts, the ISEMFs, can receive a major contribution from the BM in the colons and small intestines of both mice and humans. I believe that this is the first report that BM cells can contribute to such a highly organised system of myofibroblasts as the ISEMFs.

3.6.1) Evidence for a BM origin of myofibroblasts

The origin of the myofibroblasts in the gastrointestinal tract is not clear (section 1.3.2), with reports of transdifferentiation between other mesenchymal cell types (Gabbiani, 1996; Ronnov-Jessen et al., 1995), and claims of a myofibroblast stem cell population in the lamina propria near the base of the crypt (Bockman and Sohal, 1998; Marsh and Trier, 1974a; Marsh and Trier, 1974b; Mayer-Proschel et al., 1997; Pascal et al., 1968; Sappino et al., 1989). There have been several reports that myofibroblasts in other tissues can derive from the BM. For example, Grimm et al., (Grimm et al., 2001) showed that approximately 30% of myofibroblasts in the kidney were of extra-renal origin. Although BM was not specifically documented as the origin of these cells, later observations strongly suggest that renal interstitial myofibroblasts can be so derived (Poulsom et al., 2001). There is emerging evidence to show that BM-derived MSCs have the potential to differentiate to form myofibroblasts in culture. For example, myofibroblasts will differentiate from human MSCs when they are co-cultured with colorectal carcinoma cell lines (Emura et al., 2000) or dermal fibroblasts (Ball et al., 2004). Furthermore, isolated human MSCs demonstrate characteristic myofibroblast antigen expression of SMA and vimentin, and can produce extracellular matrix proteins *in vitro* (Kadner et al., 2002).

The data obtained from this part of the project clearly suggests that a large proportion of the intestinal myofibroblasts are derived from the BM in the mouse and human small intestine and colon. This has been supported by results from subsequent studies that have shown a BM origin of myofibroblasts in the mouse lung, stomach, oesophagus, skin, kidney and adrenal glands (Direkze et al., 2003), and in a mouse model of pancreatic insulinoma, approximately 25% of all myofibroblasts descend from transplanted BM. In the human, transplanted BM cells can form up to 45% of the myofibroblasts present in liver fibrosis (Forbes et al., 2004).

3.6.2) Contribution of BM stem cell subpopulations to myofibroblasts

Previous studies demonstrating BM engraftment in the mouse and human gastrointestinal tract have used either whole BM, or purified populations of HSCs or MAPCs (Jiang et al., 2002; Korbling et al., 2002; Krause et al., 2001; Okamoto et al., 2002; Spyridonidis et al., 2004). Although these studies report a low level of epithelial cell engraftment within the gastrointestinal tract, they do not observe BM colonisation of the pericryptal sheath. The reasons for these inconsistencies are unclear, although species differences, discrepancies in the number and phenotype of transplanted cells, detection techniques and time elapsed between BM transplant and analysis may influence the results. Reports of myofibroblasts of BM origin in other tissues have all been based upon the transplantation of whole BM (Direkze et al., 2003; Forbes et al., 2004; Poulsom et al., 2001), and therefore cell sorting experiments are essential to locate the defined ISEMF progenitor cells of the BM.

3.6.3) BM forms cellular columns of ISEMFs in the pericryptal sheath

Under normal circumstances, pericryptal myofibroblasts proliferate and migrate upward along the crypt-villus axis until they are shed into the intestinal lumen (Kaye et al., 1968; Parker et al., 1974; Pascal et al.,

1968). Following irradiation in the rodent and human gastrointestinal tract, there is prominent loss of epithelial cells, and the pericryptal myofibroblasts also undergo apoptosis on a time-scale very closely associated with that seen in the epithelial cells (Thiagarajah et al., 2000; Wiernick and Perrins, 1975). The observations of rows of Y chromosome-positive ISEMFs indicate that transplanted BM cells can contribute to the turnover of the pericryptal myofibroblast sheath, presumably due to the regenerative demand incurred by irradiation damage at the time of BM transplant (Naftalin, 2004). These interconnected columns of BM-derived ISEMFs are suggestive evidence for clonal expansion, and I postulate that BM cells initially seed the lamina propria near the crypt base, and proliferate to produce daughter cells, which migrate upward along the crypt-villus axis. The extension of these cellular columns of donor-derived ISEMFs to the tip of the villi of the small intestine supports this theory of continuous migration by BM-derived cells.

Contrary to the results of earlier studies showing an upward migration of pericryptal myofibroblasts along the crypt-villus axis, Neal and Potten (Neal and Potten, 1981) suggested that intestinal myofibroblasts travel laterally in the lamina propria, and found no evidence of vertical migration of these cells in the mouse small intestine. The observations of BM-derived cellular columns may be explained simply by the engraftment of a number of transplanted cells in the lamina propria, and markers of proliferation are vital to unequivocally demonstrate the clonogenic status of these cells.

Unlike in the mouse colon, rows or clusters of BM-derived cells in the lamina propria of the human tissue were not observed, and the numbers of BM-derived ISEMFs varied widely between individual patients although were far less abundant than in the mouse. I experienced considerable difficulty in accurately quantifying the number of Y chromosome-positive cells in both the control male tissues and in the experimental transplant tissues, which may reflect differences in the degree of intestinal damage in each patient and/or technical factors such as tissue fixation.

3.6.4) BM-derived ISEMFs contribute to the intestinal epithelial stem cell niche

The pericryptal myofibroblast sheath is closely associated with the epithelial cells of its crypt and is thought to influence epithelial behaviour by producing a number of growth factors and cytokines, the receptors for which are often located on the epithelial cell membrane (section 1.3.2; reviewed in (Powell et al., 1999a; Powell et al., 1999b)). Stem cells within many different tissues are believed to reside within a niche, which provides the optimal microenvironment for the stem cells to produce their differentiated progeny (section 1.2; (Schofield, 1978)). The epithelial cells in the lower regions of the intestinal crypts are the postulated stem cells, and the subjacent ISEMFs and their secreted basement membrane factors are proposed to form and maintain the stem cell niche. It is well documented that ISEMFs influence epithelial cell proliferation and regeneration through epithelial: mesenchymal crosstalk, and ultimately determine epithelial cell fate. BM-derived ISEMFs were frequently observed surrounding the epithelial cells in the base of the intestinal crypts, and I therefore propose that BM may contribute to the cells of the intestinal stem cell niche. This indicates that BM cells may have an indirect function in regulating intestinal epithelial cell proliferation, *via* the paracrine secretion of specific growth factors and cytokines.

3.6.5) Microchimerism

Microchimerism is defined as a small population of cells or DNA from one individual that is harboured in another individual. For example, during pregnancy cells can traffic between the foetus and the mother, and low levels of fetal cells have been shown to persist in the maternal circulation for up to 27 years postpartum (Bianchi et al., 1996). Persistent microchimerism can also follow a blood transfusion, or can occur from cell transfer between twins *in utero* (reviewed in (Lambert and Nelson, 2003)).

In a recent study by Spyridonidis et al. (Spyridonidis et al., 2004), showing BM-derived colonocytes in humans, microchimerism was considered by investigating the presence of a Y chromosome in colonocytes in colonic biopsies from female patients that had received a sex-matched BM transplant. A Y chromosome was not observed in cells in any of the tissue biopsies, despite 5 of the patients having a history of male childbirth, and all patients having a history of recent blood transfusion. This suggests that microchimerism may be very rare, although it is important to consider all possible origins of the donor-derived cells. In the murine studies there is no doubt that the BM was the origin of the new ISEMFs, although in the human tissues it was not possible to establish unequivocally that the donor BM is the source of the ISEMFs as the patients' history of childbirth and blood transfusion were not known.

3.7) Conclusion

Based upon the results of this part of the study, I conclude that an ISEMF progenitor population exists in the BM, which can contribute to the repair of the pericryptal sheath following irradiation. ISEMFs of donor origin were observed up to 6 weeks post-transplant when the intestinal mucosa and pericryptal sheath have fully regenerated, which suggests that BM-derived cells are sustained within the intestine. I suggest that BM-derived myofibroblasts contribute to the intestinal epithelial stem cell niche, and therefore can indirectly influence epithelial stem cell behaviour. I also speculate that BM-derived ISEMFs can proliferate and migrate upward along the crypt:villus axis, although this remains to be proven. Therefore, cells in the BM could potentially be used as vehicles for gene delivery to the gut, to modify both myofibroblast and epithelial cell behaviour.

Table 3.1) BM contribution to ISEMFs in mouse colon at defined timepoints after whole BM transplantation.

Time post-BM transplant	BM-derived ISEMFs [a]
14 days (n = 6)	49.5 ± 2.8
6 weeks (n = 5)	57.6 ± 4.4 [b]

[a] Mean ± standard error of the mean percentage of ISEMFs that were BM-derived.

[b] Non-significant variances in mean percentage of ISEMFs at 14 days compared to 6 weeks ($p = 0.103$).

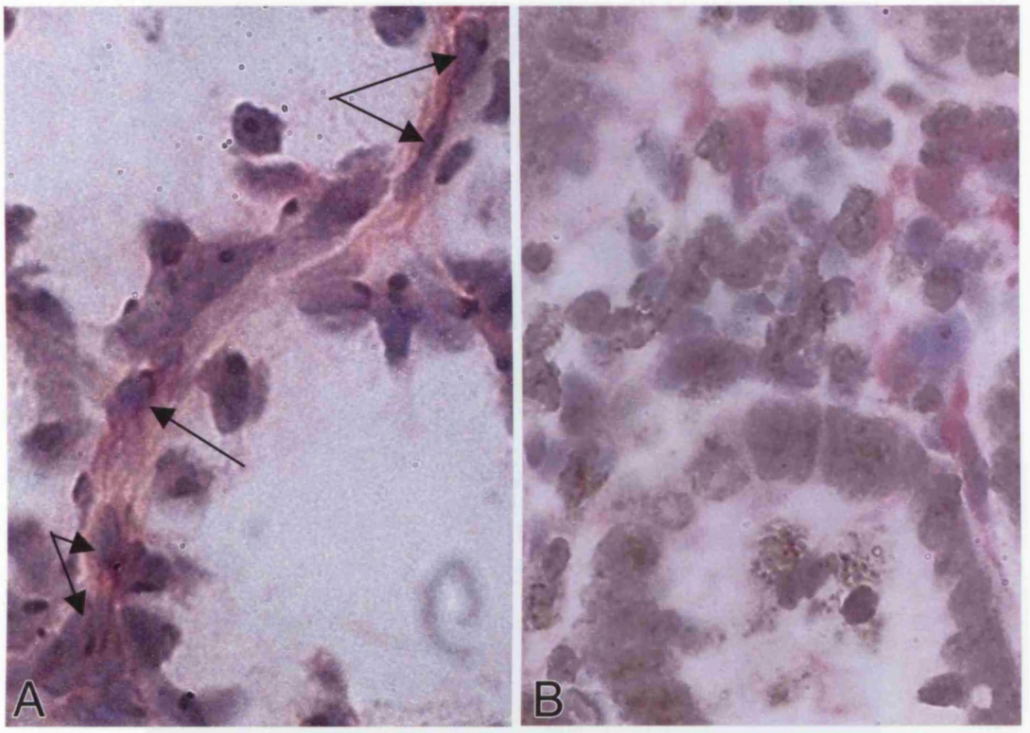

Figure 3.1) *In situ* hybridisation for the Y chromosome in male and female wild type mouse controls.

Following *in situ* hybridisation for a Y chromosome-specific probe, the Y chromosome is identified as a dark brown spot in the cell nucleus. Subepithelial intestinal myofibroblasts (ISEMFs) were identified by their morphology, their location in the lamina propria, and by their immunoreactivity for smooth muscle actin (SMA; red cytoplasmic stain). Approximately 69.2% of all ISEMFs contained a Y chromosome in the male colons (A; arrows), and ISEMFs in female colons lacked Y chromosome expression (B).

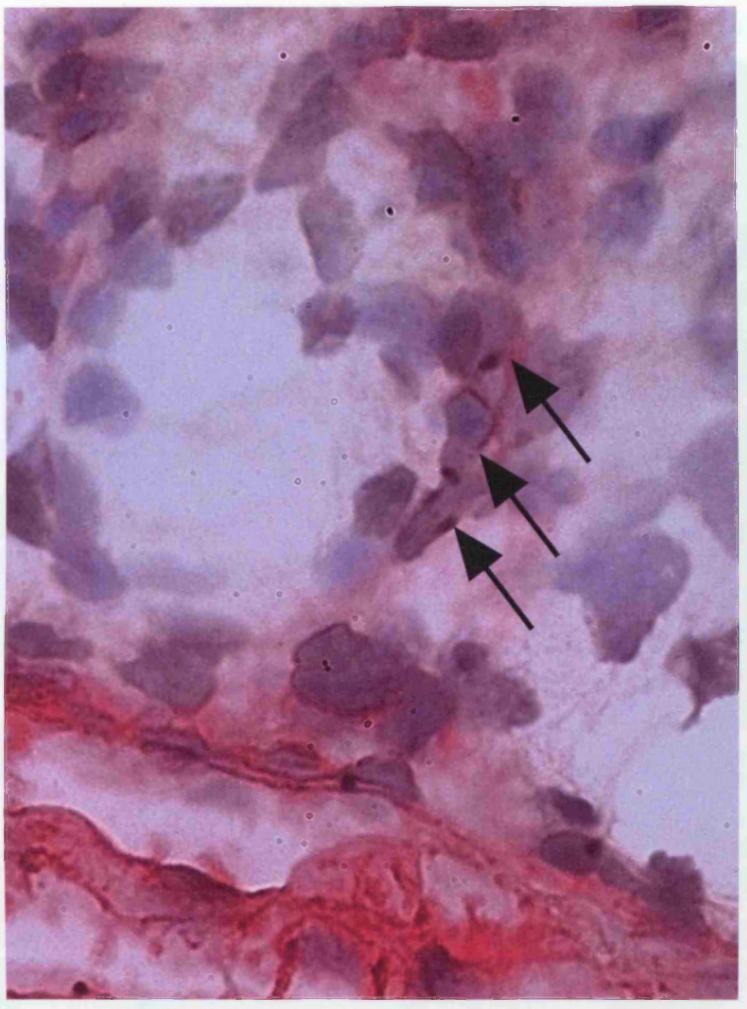

Figure 3.2) BM-derived ISEMFs are present 7 days post-transplant. As early as one week post-transplant, a few cells were present in the subepithelial lamina propria of the mouse small intestine and colon that were of BM origin. This figure shows Y chromosome-expressing ISEMFs in the female mouse colon at this early timepoint (arrows).

Figure 3.3

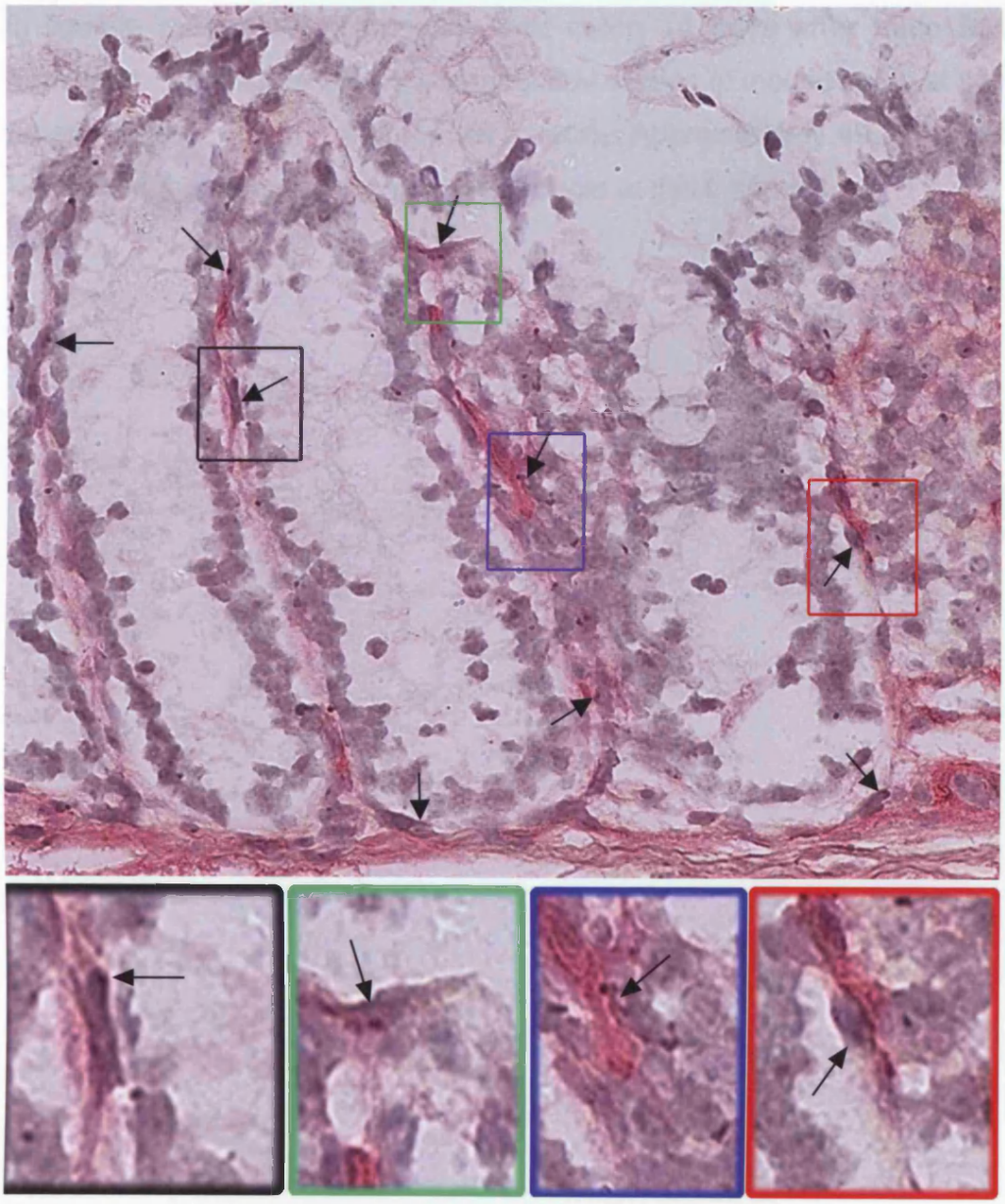

Figure 3.3) BM-derived ISEMFs are present 14 days post-transplant. Numerous Y chromosome-positive cells that are immunoreactive for SMA, and are therefore believed to be ISEMFs of BM origin are present in female mouse small intestine and colon 14 days after male BM transplant. This figure shows a longitudinal section of mouse colon at this timepoint (e.g., arrows; high power insets). Approximately 49.5% of all ISEMFs in the mouse colon are of BM origin at this timepoint.

Figure 3.4

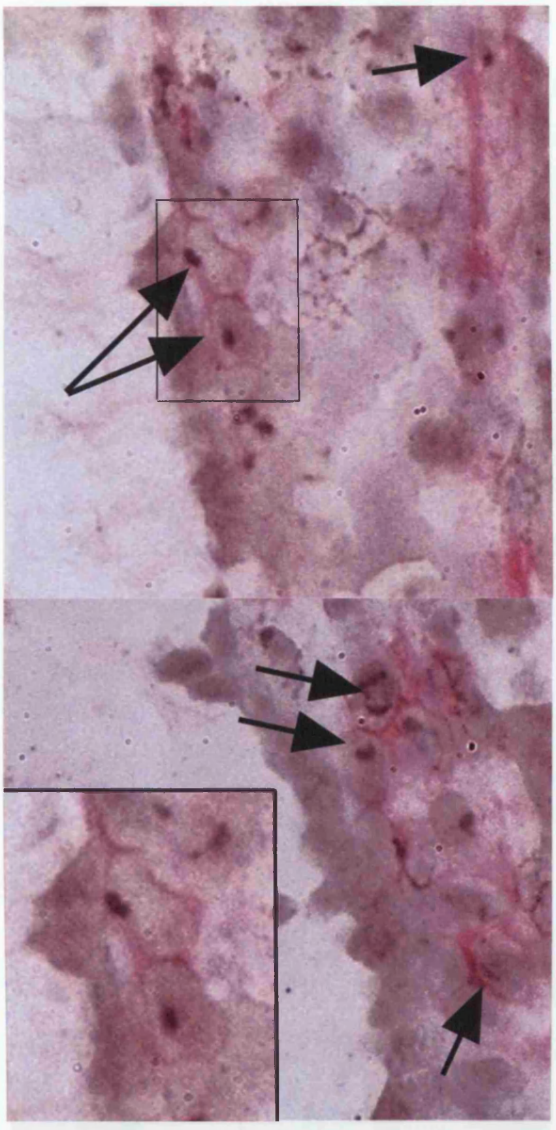

Figure 3.4) ISEMFs in the lamina propria are frequently derived from the BM.

In female mice, transplanted with whole BM from male donors, approximately 57.6% of ISEMFs in the colon are of donor origin, 6 weeks after transplant (e.g. arrows; high power inset).

Figure 3.5

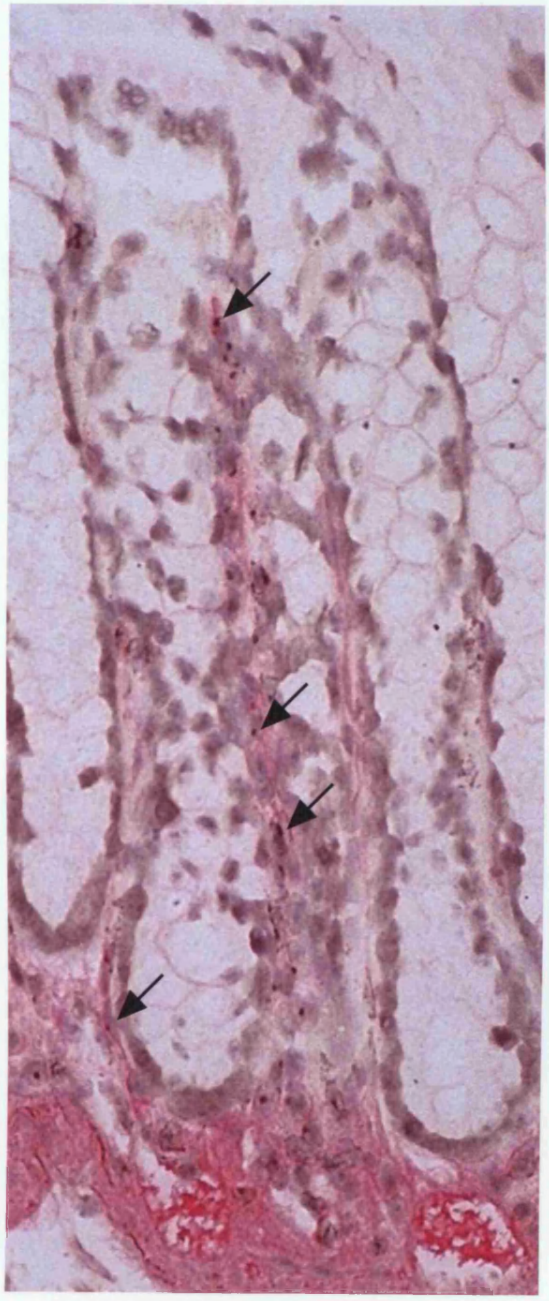

Figure 3.5) BM-derived ISEMFs are often present in cellular columns in the subepithelial lamina propria

Y chromosome-positive ISEMFs stained with SMA in the mouse colon at 6 weeks post-transplant. These cells were frequently present in rows, extending from the base of the crypt upward to the intestinal lumen (arrows).

Figure 3.6

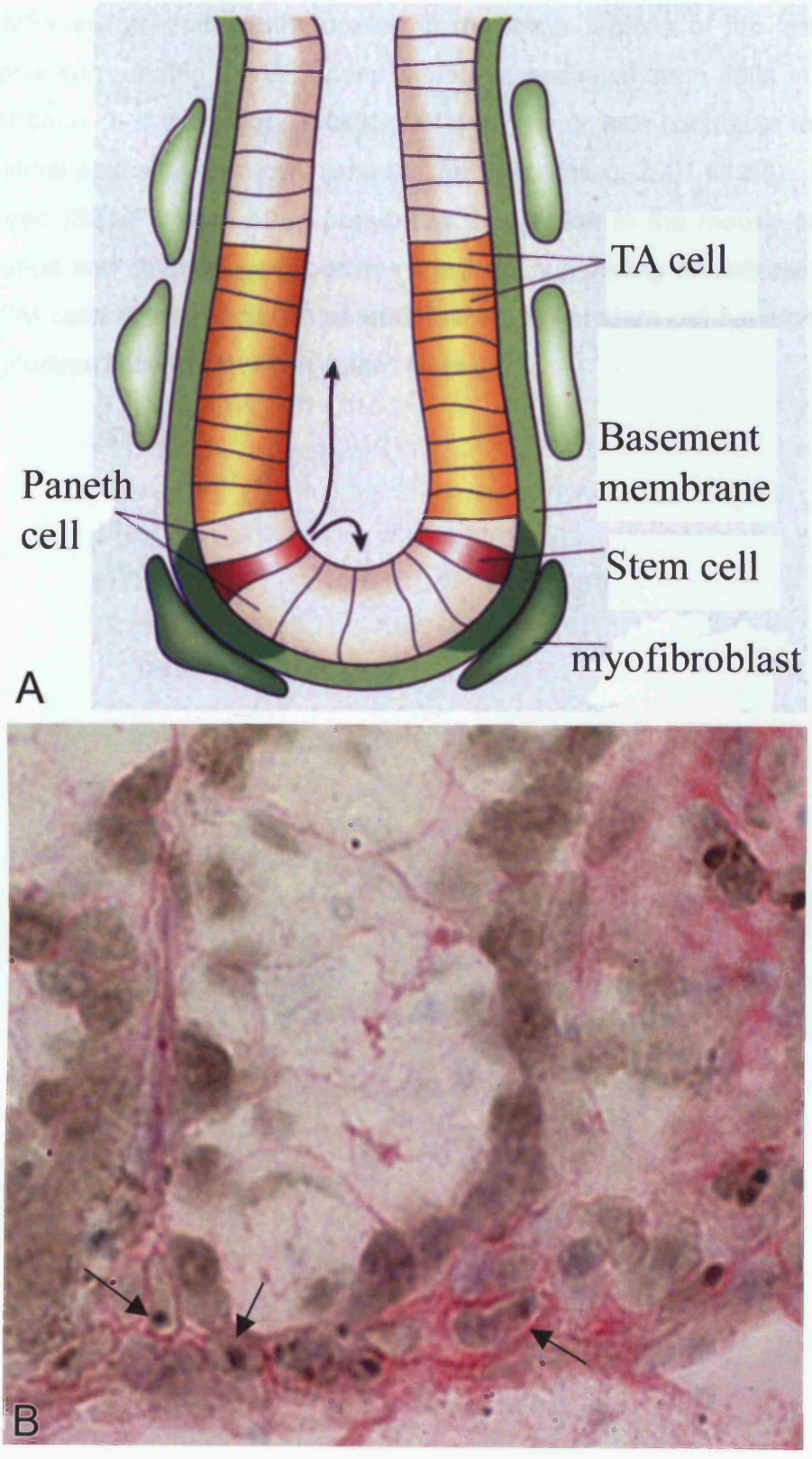
TA cell
Basement membrane
Paneth cell
Stem cell
myofibroblast
A
B

Figure 3.6) BM-derived ISEMFs may contribute to the intestinal epithelial stem cell niche.

ISEMFs are predominantly located in the lower regions of the lamina propria surrounding the proposed intestinal epithelial stem cells in the crypt base. It is therefore postulated that myofibroblasts contribute to the intestinal epithelial stem cell niche (A; from {Spradling, 2001 #129}). BM-derived ISEMFs were often present in this region in the mouse small intestine and colon, 6 weeks after transplant, suggesting an indirect role for BM cells in the regulation of intestinal epithelial stem cell function (B; longitudinal section of mouse colon; arrows).

Figure 3.7

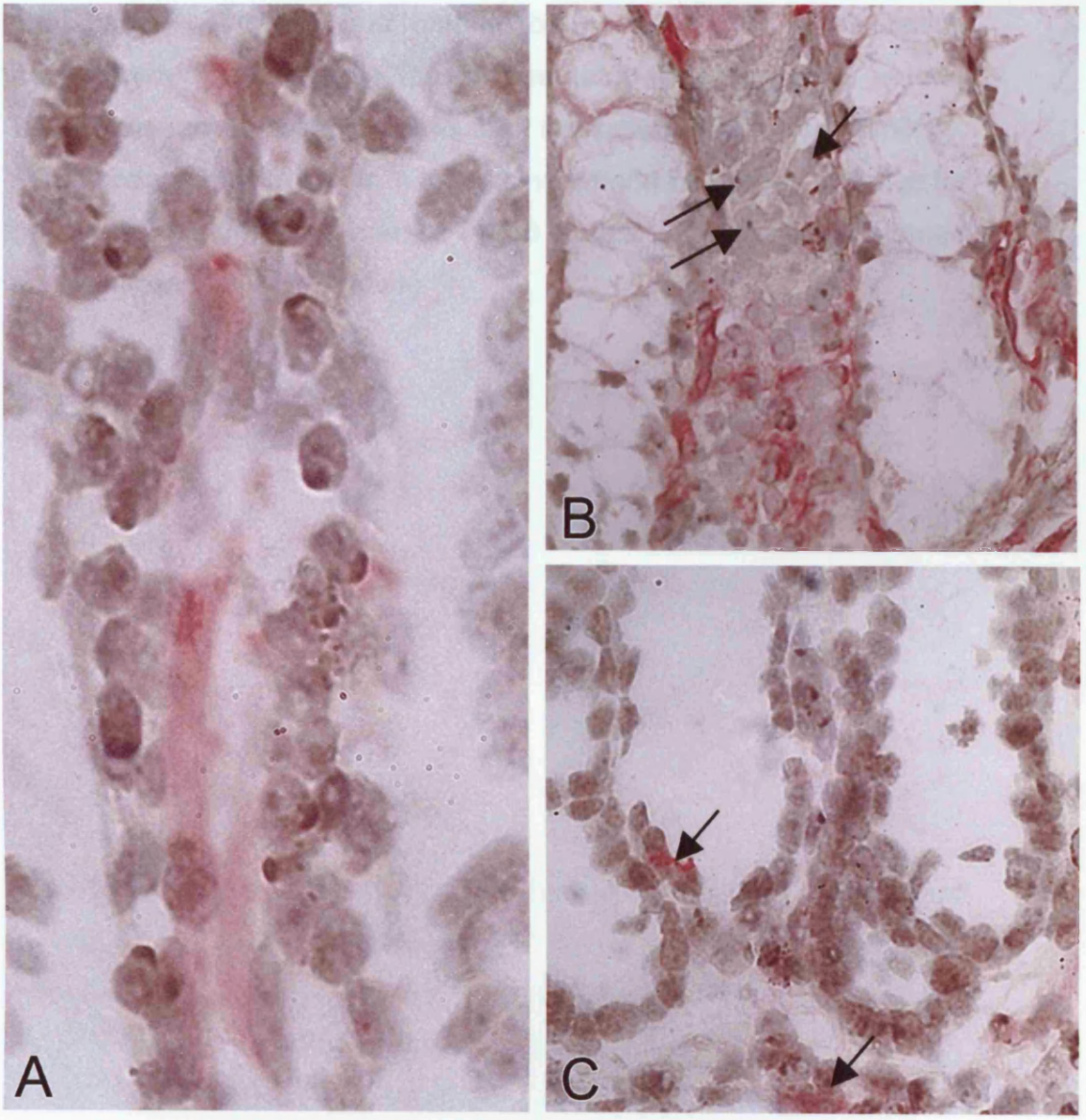

Figure 3.7) Some BM-derived cells do not express desmin, or haematopoietic or macrophage markers.

Desmin-immunoreactive cells within the lamina propria were not Y chromosome-positive (A). Y chromosome-positive cells in the lamina propria were often negative for the haematopoietic progenitor marker CD34 in the mouse colon, 6 weeks after transplant (B; arrows). Similarly, Y chromosome-positive cells in the lamina propria were often negative for the mouse macrophage marker F4/80 antigen (C; positively stained macrophages shown by arrows).

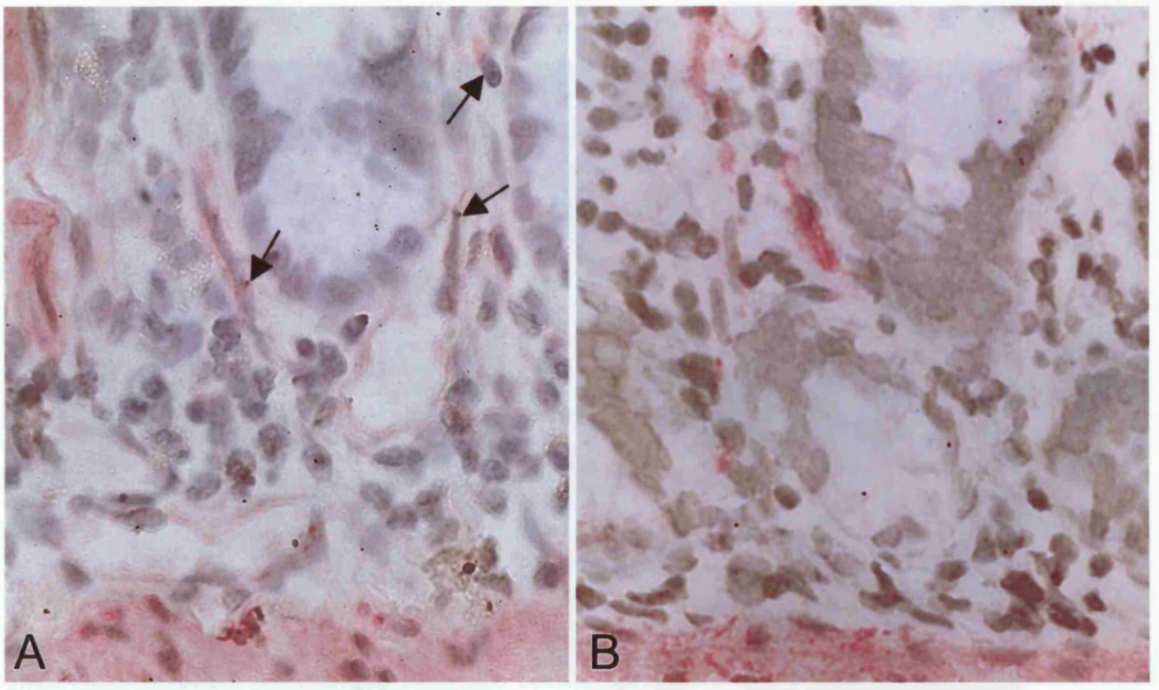

Figure 3.8) *In situ* hybridisation for the Y chromosome in male and female human control intestines.

Following *in situ* hybridisation for a Y chromosome-specific probe, the Y chromosome is identified as a dark brown spot in the cell nucleus. Subepithelial intestinal myofibroblasts (ISEMFs) were identified by their morphology, their location in the lamina propria, and by their immunoreactivity for SMA (red cytoplasmic stain). This figure shows Y chromosome-expressing ISEMFs in the male duodenum (A; arrows), and ISEMFs in female duodenal control tissue that lacked Y chromosome expression (B).

Figure 3.9

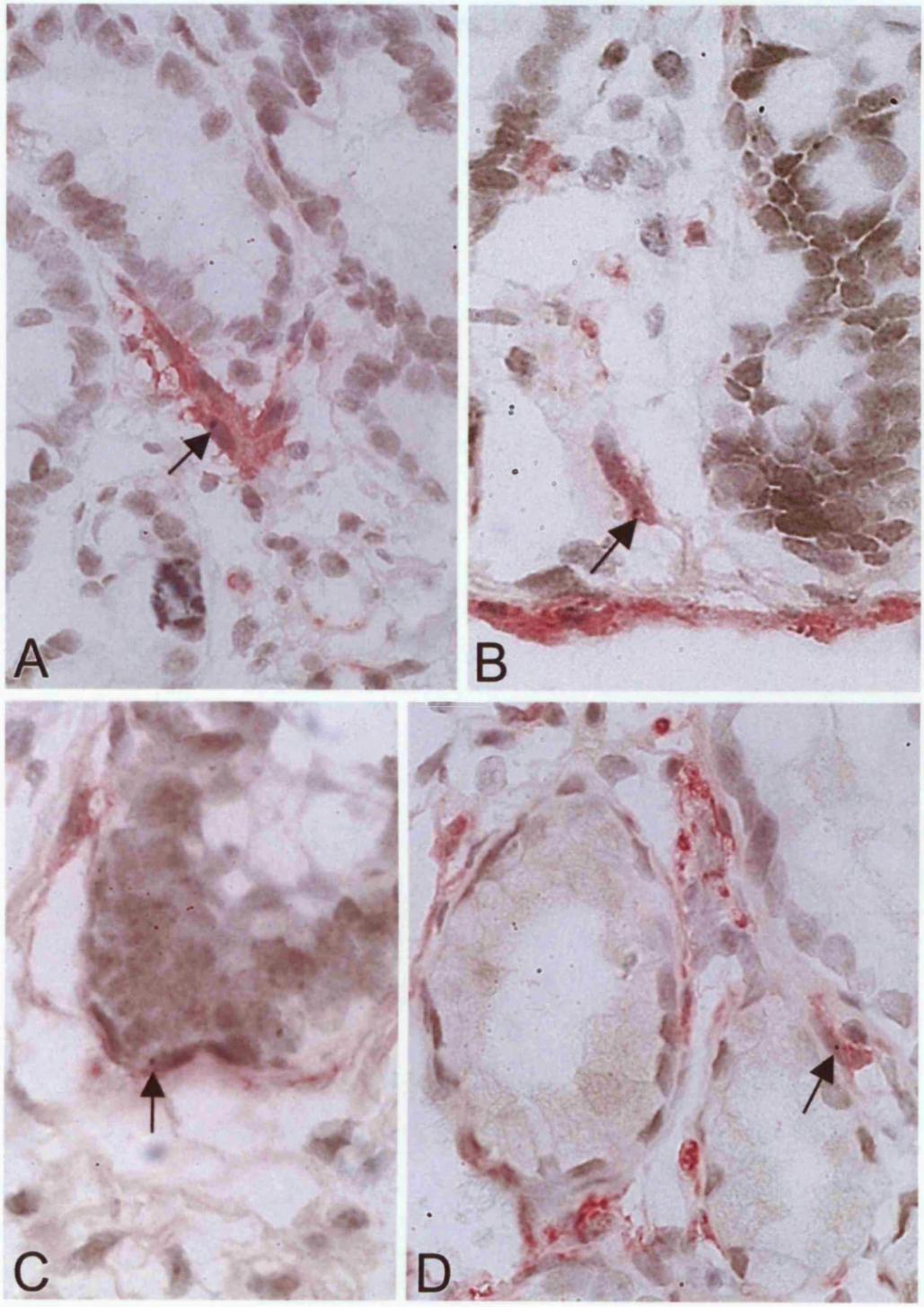

Figure 3.9) BM-derived ISEMFs in the human gastrointestinal tract.
Human gastrointestinal biopsies from female patients who have received a BM transplant from a male donor, showing ISEMFs that are immunoreactive for SMA and positive for the Y chromosome probe (A – D; arrows).

CHAPTER IV

In experimental colitis, the input of BM-derived ISEMFs is significantly increased, and BM plays an additional major role in neovasculogenesis.

4.1) Introduction

As discussed in Chapter III, following lethal irradiation and whole BM transplantation, a significant number of ISEMFs of donor origin were observed in the pericryptal sheath of the lamina propria in the mouse and human small intestine and colon. True to the nature of the indigenous ISEMFs, BM-derived ISEMFs are believed to form and maintain the intestinal epithelial stem cell niche, and thus contribute to the regulation of epithelial cell proliferation via mesenchymal:epithelial cell interactions. Therefore, BM-derived ISEMFs may have an indirect role in the maintenance of intestinal homeostasis by regulating epithelial stem cell turnover (Brittan et al., 2002).

4.1.1) BM plasticity is enhanced with increasing regenerative demand

The contribution of BM cells to non-haematopoietic lineages in adult tissues is upregulated with increasing regenerative pressure induced by injury or disease (section 1.13). However, prior to this study, the role of BM cells in tissue remodelling in the inflamed gut was not known.

4.1.2) ISEMFs are activated in IBD

In the healthy gut, ISEMFs exist as transiently differentiated, quiescent cells. However, in inflamed tissue, they are activated to increase their

proliferation rate (McKaig et al., 2002), upregulate their expression of cytokines, chemokines, growth factors and adhesion molecules, and to enhance their secretion of soluble mediators of inflammation and extracellular matrix factors (reviewed in (Powell et al., 1999a)). For example, activated ISEMFs increase their secretion of the epithelial cell mitogen, keratinocyte growth factor in IBD, thereby stimulating epithelial cell hyperplasia (Bajaj-Elliott et al., 1997). Furthermore, the pro-inflammatory cytokines interleukin-1β and tumour necrosis factor-α (TNFα) are upregulated in Crohn's disease (Mahida et al., 1989; Reinecker et al., 1993), and these have been shown to drive the proliferation of human colonic myofibroblasts *in vitro* (Jobson et al., 1998). It is well documented that overactivation and persistence of ISEMFs in Crohn's disease can result in tissue fibrosis and scarring (Aigner et al., 1997; Graham, 1995; Isaji et al., 1994; Kinzler and Vogelstein, 1998; Martin et al., 1996), which indicates that the pathways of tissue regeneration by ISEMFs in Crohn's disease are stringently controlled and can prove deleterious if dysregulated.

4.1.3) BM contributes to vascular lineages in inflamed and diseased tissues

Endothelial progenitor cells (EPCs) originating in the adult BM are involved in the remodelling of existing blood vessels by angiogenesis, and in the formation of new blood vessels by postnatal vasculogenesis, thereby playing a role in vascular repair and remodelling in specific physiological and pathological conditions (Asahara et al., 1997; Grant et al., 2002; Reyes et al., 2002; Sata et al., 2002; Shi et al., 1998). Endothelial cells and their surrounding vascular smooth muscle (mural) cells derived from EPCs have been reported in blood vessels in various ischaemic organs and tissues in both mice and humans (see Table 1.1). However, to date, there have been no reports of BM-derived vascular lineages in the colon, and the role of BM in tissue regeneration in IBD is poorly understood.

4.2) Aim

In order to investigate the therapeutic relevance of BM cells in intestinal regeneration, we used the 2,4,6-trinitrobenzene sulfonic acid (TNBS) model of experimental colitis, a well-established model of T helper cell type I-mediated gut inflammation, with similarities to human Crohn's disease (Neurath et al., 1995). This model of intestinal inflammation was selected as it directly involves the ISEMFs, which we have confirmed can be derived from a population of cells in the BM (Brittan et al., 2002). Emerging data indicates that BM plasticity is enhanced in diseased tissues, although this has not yet been demonstrated in a model of IBD. Furthermore, increasing evidence suggests that transplanted BM cells can provide long-term remission of Crohn's disease, which is as yet incurable, although the mechanism by which the BM cells alleviate colitis is poorly understood.

We now show that in TNBS colitis, transplanted BM cells significantly enhance their engraftment into the damaged colon, and give rise to ISEMFs of donor origin. Furthermore, we demonstrate that BM cells can form both endothelial and mural cells in blood vessels in the colon, again with a significant increase in colitis. Blood vessels were observed that were composed entirely of cells of BM origin, highlighting the possible role of BM in neovasculogenesis.

4.3) Methods

Female Balb/C donor mice were given a lethal dose of irradiation followed by a whole BM transplant from male Balb/C donor mice (section 2.1). Six weeks post-transplant, experimental colitis was induced in the mice by intrarectal injection of 2 mg TNBS in 50% ethanol. Control animals were given an injection of 50% ethanol only. Animals were killed and colons were fixed and processed for analyses at 1, 4, 7, 8 and 14 days post-TNBS. Donor male cells were detected by *in situ* hybridisation with a Y chromosome-specific probe, which was combined with immunohistochemistry for specific antigens to determine the phenotype of the donor cells (section 2.3). Male and female mouse colons were used as positive and negative controls, respectively, for the *in situ* hybridisation protocol.

A more detailed description of the materials and methods used in this chapter is given in Chapter II, section 2.2.2.

4.4) Results

4.4.1) TNBS-induced colitis

Previous studies have shown that TNBS-induced colitis is manifest as patchy lesions in the distal colon that gradually worsen throughout days 1 - 8 (Higgins et al., 1999). Examination of H & E stained paraffin sections of the colons at all timepoints after TNBS administration, confirmed earlier reports and allayed our initial concerns that the pathology of TNBS colitis may be altered due to irradiation and BM reconstitution. Affected areas showed an acute transmural disease with inflammatory cell infiltration, oedema, ulceration, fibrosis, epithelial cell necrosis and loss of colonic crypts. Fourteen days after TNBS administration, the colons had almost entirely returned to normal (Figure 4.1A – E).

4.4.2) Identification of BM-derived cells by *in situ* hybridisation

As paraffin sections analysed by *in situ* hybridisation for Y chromosome-specific sequences were 4 μm thick, the Y chromosome was not present in all nuclear profiles and the observed proportion of donor-derived cells in female colons following a male BM transplant was therefore underestimated. Approximately 69.2% of cell nuclei in colons from male control mice show a Y chromosome using the *in situ* hybridisation technique. Therefore, the observed values of donor cells in the female recipient tissues were divided by a factor of 0.692 to reflect total BM-derived cell populations.

4.4.3) BM-derived ISEMFs in ethanol-treated control colons

At all time-points, Y chromosome-positive cells were abundant throughout the lamina propria in colons of female control mice that received a male BM transplant and were given 50% ethanol only. Based upon previously

established criteria, an ISEMF phenotype was attributed to those donor-derived cells that had a spindle-shape, resided in the subepithelial lamina propria, were immunoreactive for SMA (Figure 4.2A) and did not express the mouse macrophage marker F4/80 or the haematopoietic progenitor marker CD34 (section 3.4.3; (Sappino et al., 1990; Schmitt-Graff et al., 1994)). Throughout the time-course, the contribution of transplanted BM to ISEMFs in this ethanol control group remained relatively constant (Table 4.1). Leucocytes throughout the lamina propria were also Y chromosome-positive, as expected.

4.4.4) BM-derived ISEMFs in undamaged mucosa of mice given TNBS

Due to the patchy nature of TNBS colitis, regions of lamina propria that displayed a typically normal morphology and no significant inflammation were assessed separately from the adjacent patches of colitis. In these regions of undamaged mucosa, donor-derived ISEMFs were identified at all timepoints (Figure 4.2B,C). In both ethanol-treated control colons and in the regions of undamaged mucosa in TNBS-treated animals, the ISEMFs were often present as rows of cells extending from the base of the crypt to the intestinal lumen, consistent with our previous observations (Figure 4.3; section 3.4.5). Approximately 24% of ISEMFs in the undamaged mucosa were derived from the transplanted BM 1 - 4 days after TNBS administration, increasing to 26% by days 6 - 8 when the adjacent colitis was most severe. By day 14, parallel to the regression of colitis, the proportion and total number of donor-derived ISEMFs had decreased once more, with approximately 22% of ISEMFs having a Y chromosome (Table 4.1).

4.4.5) BM-derived ISEMFs in severely inflamed colons of mice with TNBS-induced colitis

In regions of severe tissue injury surrounding ulcer beds in TNBS-treated animals, very large numbers of cells throughout the lamina propria, muscularis mucosa, submucosa, and muscularis propria had a Y chromosome, due to the high numbers of inflammatory cells in colitis. In these regions at 6 - 8 days post-TNBS, 39% of ISEMFs were derived from the transplanted BM (Figure 4.4; Table 4.1), and interestingly, donor-derived myofibroblasts at this timepoint were three-fold more numerous than in the ethanol-treated control colons. In the lamina propria surrounding regions of re-epithelialisation, the myofibroblasts assumed an activated phenotype with a large, rounded morphology (Figure 4.4). At 14 days post-TNBS there was no evidence of colitis.

4.4.6) BM-derived cells form vascular lineages in TNBS-induced colitis

We observed BM-derived vascular mural cells, confirmed by morphological criteria and their expression of a Y chromosome and immunoreactivity for SMA, at all timepoints studied in both the ethanol controls and in TNBS-treated animals (Figure 4.5). In colons showing an apparently normal morphology, and in non-inflamed regions of colons in TNBS-treated mice, BM-derived mural cells were frequently present as single cells, interspersed throughout the blood vessels. However, in patches of severe inflammation, vessels were observed that appeared to be composed largely, and even entirely, of BM-derived cells, thereby suggesting that BM is involved in the creation of entire new blood vessels *via* postnatal neovasculogenesis (Figure 4.6). In grossly inflamed regions, the tissue was highly vascularised containing small, leaky vessels, and BM-derived mural cells were frequently present in these structures (Figure 4.6B). The numbers of blood vessels containing BM-derived mural cells were calculated, revealing a 6.4-fold increase in TNBS-treated colons than compared to ethanol-treated controls ($p = 0.013$; Table 4.2). Moreover, the total percentages of BM-derived mural cells within these blood vessels was calculated, showing an almost 2-fold

increase in the contribution of BM to mural cells in colitis, compared to ethanol-control tissue ($p = 0.017$; Table 4.2).

BM cells also contributed to endothelial cells within blood vessels in inflamed colons, ascertained by their morphology and their co-expression of ICAM-1 and the Y chromosome (Figure 4.7). However, the transition of BM to endothelial cells appeared to occur less frequently than BM formation of mural cells, as BM-derived endothelial cells occurred only in vessels in grossly inflamed tissue i.e., at 6 - 8 days post-TNBS.

4.4.7) BM-derived ISEMFs and vascular cells do not express haematopoietic or macrophage markers

Following detection of the Y chromosome-positive ISEMFs and vascular lineages, immunohistochemical staining was performed for the haematopoietic marker CD34, or the macrophage marker F4/80, to establish whether BM-derived lineages were haematopoietic or macrophage cell types. We frequently observed cells of BM origin in the blood vessels that lacked expression of F4/80 (Figure 4.8) and CD34 (Figure 4.9). Similarly, BM-derived cells were present in the lamina propria of both normal colons and in inflamed colons, which displayed a myofibroblast phenotype, but lacked expression of these markers (Figure 4.10). Macrophages or haematopoietic cells derived from the BM were present within the lamina propria and blood vessels (Figure 4.8 - 4.10) and thereby provided an internal control for this experiment.

4.5) Discussion

These data confirm my previous observations that BM forms ISEMFs in the lamina propria of the mouse gut (chapter III; (Brittan et al., 2002)). I now show that this phenomenon is significantly increased in murine colitis. I believe that this study presents a number of new observations: (1) BM contribution to ISEMFs is enhanced in colitis, (2) BM cells give rise to vascular lineages in the mouse colon and, (3) the role of BM cells in vasculogenesis is upregulated in colitis. These data indicate that BM has multiple therapeutic roles in colitis, in its production of both mesenchymal and vascular lineages that aid tissue regeneration and indirectly regulate intestinal epithelial stem cell behaviour, the implications of which will be discussed in the following sections.

4.5.1) Confirmation of a BM origin of ISEMFs in the mouse colon

I have verified my previous findings, that ISEMFs in the mouse colon are derived from a progenitor population of cells in the BM (section 3.6.1; (Brittan et al., 2002)). I also confirm my observations of cellular columns of BM-derived ISEMFs in the subepithelial lamina propria, spanning from the crypt base to the intestinal lumen (section 3.6.3; (Brittan et al., 2002)). It is tempting to speculate that this provides further evidence of a clonal nature of BM-derived ISEMFs surrounding individual crypts, although markers of cell proliferation are required to unequivocally demonstrate this. Furthermore, as expected from my previous results, numbers of BM-derived ISEMFs were high, even in apparently undamaged, non-colitic tissue, presumably due to the regenerative demand incurred by irradiation damage at the time of BM transplant (Naftalin, 2004).

4.5.2) BM formation of ISEMFs is significantly upregulated in colitis

BM contribution to adult cells in non-haematopoietic tissues is upregulated in damage or disease (section 1.13), which we can now confirm, for the first time, also occurs in the inflamed colon. The observed increase in BM engraftment in the inflamed colons demonstrates an augmented BM contribution to differentiated lineages in the intestine during times of stress, and outlines the significance of ISEMFs in tissue regeneration in IBD. However, it is not known whether increased numbers of circulating BM cells are stimulated to engraft within the diseased colon and form ISEMFs after TNBS administration, or if pre-existing BM-derived ISEMFs in the lamina propria become activated to divide and repopulate the damaged pericryptal sheath. Markers of cell proliferation could confirm the clonogenic potential of BM-derived ISEMFs.

ISEMF number is increased in Crohn's disease (Pucilowska et al., 2000), which is believed to be a result of the activation and differentiation of quiescent interstitial fibroblasts or pericytes, or the increased proliferation and/or decreased apoptosis of existing myofibroblasts (Adegboyega et al., 2002). However, my observations suggest a further contributory factor to this increase in myofibroblast number, as a substantial proportion of ISEMFs in colitis are derived from the BM.

4.5.3) BM forms multiple vascular lineages in the inflamed mouse colon

In addition to ISEMFs, we now show for the first time that transplanted BM cells form both mural cells and endothelial cells of blood vessels in the inflamed mouse colon. The "angiogenic switch" model describes the repopulation of damaged blood vessels by fully differentiated endothelial cells, which migrate to sites of injury from neighbouring blood vessels (Hanahan and Folkman, 1996). However, since the recent identification of a circulating EPC population in the BM (Asahara et al., 1997; Shi et al., 1998), evidence for postnatal neovasculogenesis has increased. The contribution of BM-derived EPCs to neovascularisation in many

inflammatory conditions is now well documented (Table 1.1), although despite this wealth of evidence, BM-derived vascular lineages have not previously been reported in IBD. Expression of the intracellular adhesion molecule, ICAM-3 (CD50), is upregulated in Crohn's disease (Bretscher et al., 2000; Fawcett et al., 1992; Serrador et al., 1997; Vainer and Nielsen, 2000), and ICAM-3 has recently been shown to be expressed on BM-derived endothelial cells and to play a regulatory role in endothelial cell junction formation (van Buul et al., 2004), possibly supporting our evidence for neovasculogenesis by BM-derived endothelial cells in colitis.

There are numerous reports of BM-derived endothelial cells, although few studies describe BM-derived mural cells (Espinosa-Heidmann et al., 2003; Orlic et al., 2001b). A recent report describes a BM origin of the periendothelial vascular mural cells, which are believed to be mural cell progenitors (Rajantie et al., 2004). It has not yet been possible to purify EPCs from the mouse BM, although it is suggested that they derive from a population of haematopoietic stem cells (HSCs) which express Flk-1, c-Kit, Sca-1, CD34, CD45, but lack markers of lineage differentiation (Lin-) (section 1.12.5; see also (Asahara and Kawamoto, 2004)). A population of mesenchymal stem cells (MSCs), termed multipotent adult progenitor cells (MAPCs), has been isolated from both murine and human BM and have also been shown to have endothelial differentiation potential, both *in vivo* and *in vitro* (section 1.12.4; (Jiang et al., 2002; Reyes et al., 2002). In our present study, since whole BM was transplanted, we cannot comment on the origins of our BM-derived cells.

4.5.4) BM-derived endothelial cells may directly influence intestinal epithelial stem cell behaviour

Paris and colleagues presented data showing that the initiating factor in radiation-induced crypt death in mice is apoptosis of the endothelial cells in the tissue stroma, which precedes intestinal epithelial stem cell dysfunction and crypt damage (Maj et al., 2003; Paris et al., 2001). The authors proposed that the endothelial cells are the primary targets of

gamma radiation, and that the concurrent death of the epithelial stem cells is a secondary event caused by a loss of endothelial cell support. However, these claims have never been corroborated and were refuted due to the lack of tissue ischaemia and hypoxia that would be expected following such severe damage to the vasculature (Hendry et al., 2001; Suit and Withers, 2001). With respect to our current findings of BM-derived endothelial cells in the mouse colon, it is important to note that if the endothelial cells in the intestine are the primary targets of radiation-induced intestinal mucosal damage this implicates the BM with a further role in intestinal epithelial stem cell regulation, *via* the formation of endothelial cells.

4.5.5) BM transplantation as a therapy for Crohn's disease

Crohn's disease runs a chronic relapsing and remitting course with a significant disease-associated mortality rate of between 6 – 24% of patients observed within a 13 – 20 year follow-up (Cooke et al., 1980; Farmer et al., 1985; Trnka et al., 1982). Crohn's disease responds to immunosuppressive medications and anti-inflammatory agents, although as yet, no curative therapy has been found. Several case studies showing long-term remission from Crohn's disease following autologous (Kashyap and Forman, 1998; Soderholm et al., 2002) or allogeneic BM transplantation (Lopez-Cubero et al., 1998; Talbot et al., 1998) have been documented. Prior to BM transplant, a patient's own BM is eradicated, and it is therefore possible that the observed remission of Crohn's disease following BM transplant is due to the elimination of aberrant BM cells that play a critical role in the pathogenesis of Crohn's disease. Furthermore, no immunosuppressive medication is required following BM transplantation in Crohn's disease patients, indicating that the observed long-term remission is a direct result of the transplantation procedure (reviewed in (James, 1998)). We now suggest a possible mechanism by which BM cells provide remission from Crohn's disease, by their formation of mesenchymal and vascular lineages in the inflamed colon, thereby contributing directly to tissue regeneration.

4.6) Conclusion

In conclusion, transplanted BM cells contribute to myofibroblasts in the lamina propria of the mouse colon in both normal and diseased states. BM engraftment and ISEMF formation is enhanced with increasing disease severity, demonstrating a possible functional role of the BM-derived ISEMFs in colitis. Transplanted BM cells also form vascular lineages in the mouse colon, again with a significant increase in colitis, and whole donor-derived blood vessels were observed, suggestive of neovasculogenesis by BM cells. It is possible that a stem cell population present in adult BM can undergo multipotential differentiation to several specialised adult cell lineages in the gastrointestinal tract, although it is equally feasible that different stem cell populations exist in the BM, each differentiating to form a specific adult lineage (Ratajczak et al., 2004).

The formation of ISEMFs and vascular lineages by BM cells in the mouse colon shows that BM cells may have multiple indirect influences on epithelial stem cell behaviour, as both these differentiated intestinal lineages are known to regulate epithelial stem cell function (Bajaj-Elliott et al., 1997; Paris et al., 2001; Powell et al., 1999b).

Our results suggest a therapeutic potential for BM cells in the formation of mesenchymal and vascular lineages, which aid tissue regeneration in Crohn's disease. However, the underlying mechanisms of BM transformation to differentiated cell lineages within extraneous tissues requires further detailed understanding before such stem cell therapy is used in a clinical environment.

Table 4.1) BM contribution to ISEMFs in ethanol-treated colons, in the normal-appearing mucosa adjacent to colitis in TNBS-treated colons, and in areas of colitis.

	EtOH control[a]	Normal mucosa adjacent to colitis[a]	TNBS colitis[a]
Days 1 - 4 (n = 7)	17 ± 1	24 ± 2.6	34.7 ± 1.3
Days 6 - 8 (n = 9)	20 ± 1.3	26 ± 1.3[b]	38.7 ± 3[c,d]
Day 14 (n = 7)	19 ± 1.2	22 ± 0.8	No colitis

[a] Mean ± standard error of the mean percentage of ISEMFs that were BM-derived

[b] Significant increase in mean percentage of BM-derived ISEMFs in normal mucosa adjacent to regions of colitis in TNBS-treated animals compared to ethanol-treated controls to at 6 - 8 day timepoint ($p = 0.012$).

[c] Significant increase in mean percentage of BM-derived ISEMFs in TNBS colitis compared to ethanol-treated control colons at 6 - 8 day timepoint ($p = 0.0095$).

[d] Significant increase in mean percentage of BM-derived ISEMFs in TNBS colitis compared to normal mucosa adjacent to regions of colitis in TNBS-treated animals, at 6 - 8 day timepoint ($p = 0.013$).

Table 4.2) BM contribution to mural cells in normal colons compared to colitis, 6 - 8 days after ethanol or TNBS administration.

	Blood vessels (%) containing BM-derived mural cells [a]	BM-derived mural cells (%) in blood vessels [b]
EtOH Control (n = 5)	4.2 ± 0.9	14 ± 3.1
Normal mucosa in TNBS-treated mice (n = 9)	15 ± 2.6	20 ± 3.6
TNBS Colitis (n = 9)	27 ± 6 [c]	27 ± 1 [d]

[a] Mean ± standard error of the mean percentage of blood vessels containing BM-derived mural cells

[b] Mean and ± standard error of the mean percentage of mural cells derived from the BM in blood vessels

[c] Statistically significant increase in percentage of blood vessels containing BM-derived mural cells in ethanol controls, compared to regions of colitis ($p = 0.013$).

[d] Statistically significant increase in percentage of total BM-derived mural cells in ethanol controls compared to regions of colitis ($p = 0.017$).

Figure 4.1

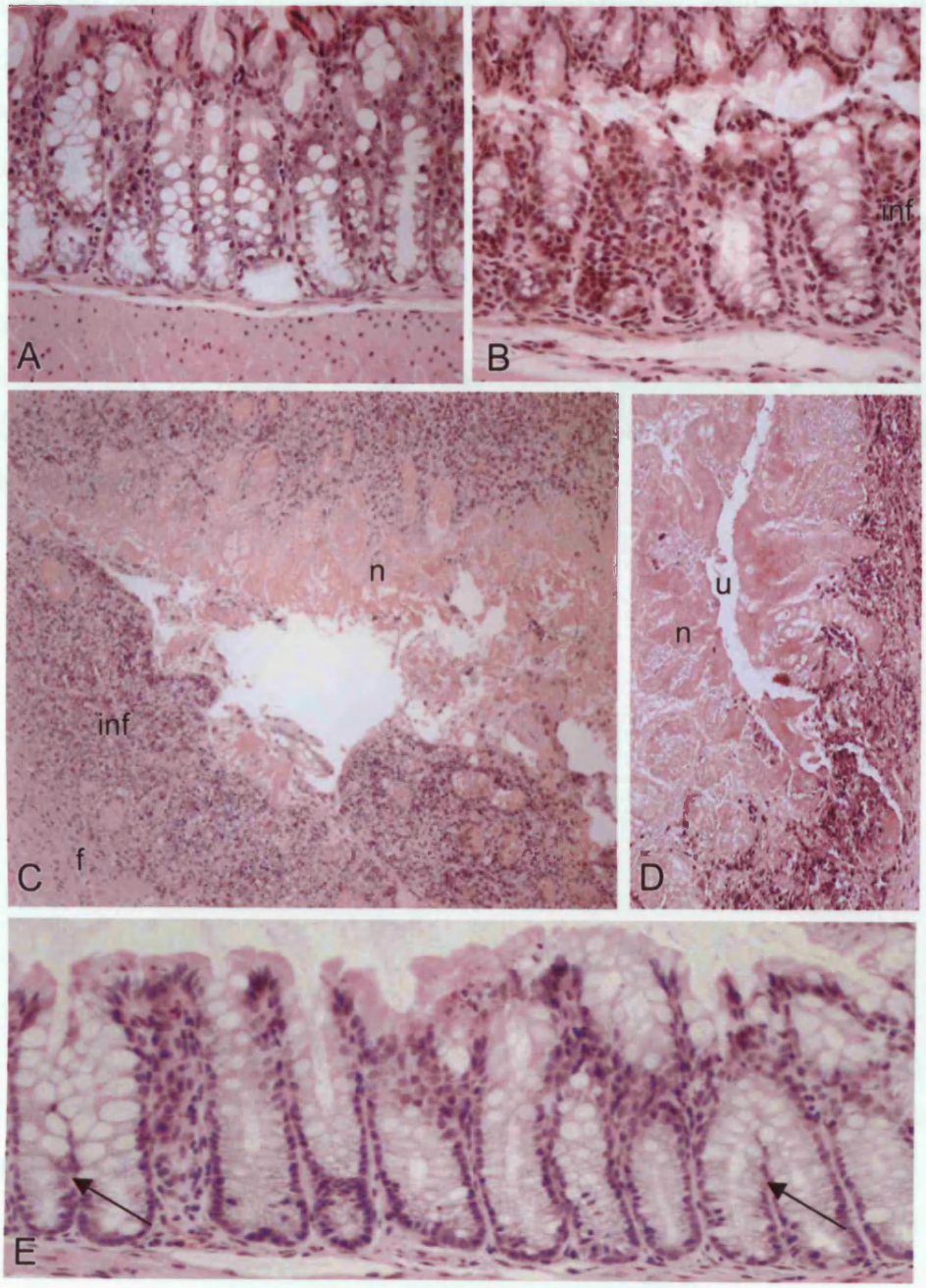

Figure 4.1) Morphological characteristics of TNBS-induced colitis. Haematoxylin and eosin-stained paraffin sections of colons at different timepoints following ethanol or TNBS administration, demonstrating the progression of TNBS-induced colitis in irradiated, BM reconstituted mice. Four days after ethanol administration, crypts appear to have a normal morphology (A). One day after TNBS, colons are relatively normal with some increased inflammation (B). At 8 days post-TNBS, colitis is most severe (C, D). By day 14 post-TNBS, the tissue has almost entirely regenerated and displays a normal morphology, with evidence of crypt fission, a key component of the regeneration process (E; arrows). inf, inflammatory cell infiltration; u, mucosal ulceration; f, fibrosis; n, epithelial cell necrosis and crypt death.

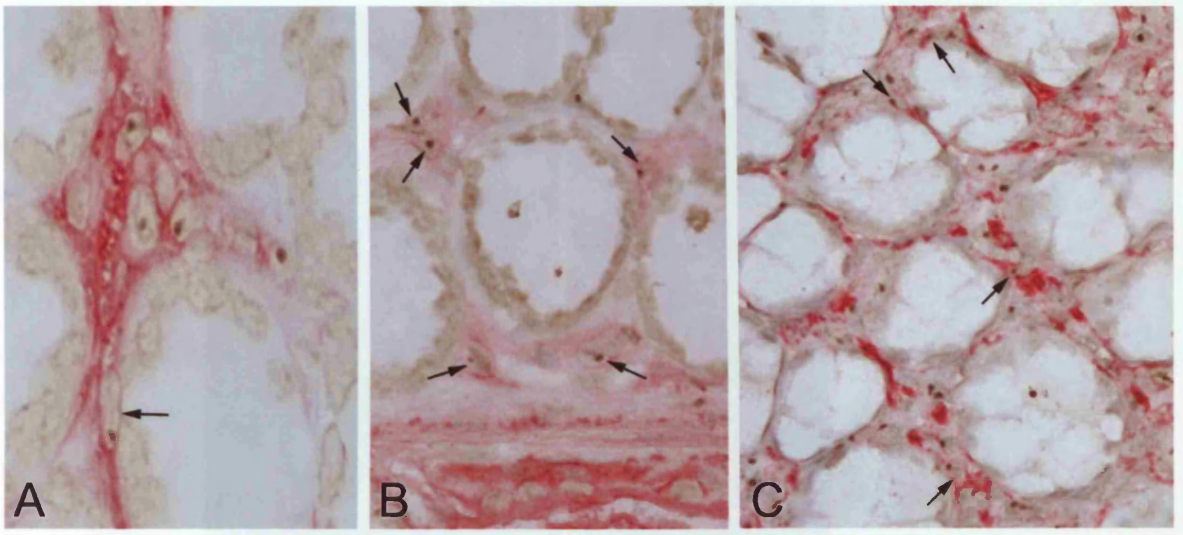

Figure 4.2) BM-derived ISEMFs in the non-inflamed mouse colon.
BM-derived ISEMFs were identified by morphological criteria i.e., spindle-shaped cells in the lamina propria surrounding epithelial crypts, which have a nuclear Y chromosome (dark brown nuclear spot), and are immunoreactive for SMA (red cytoplasm). These cells were frequently identified at all timepoints in both ethanol-treated controls (A; arrow; 4 days post-ethanol) and in the non-inflamed mucosa adjacent to regions of colitis in TNBS-treated mice (B, C; arrows; 8 days post-TNBS).

Figure 4.3

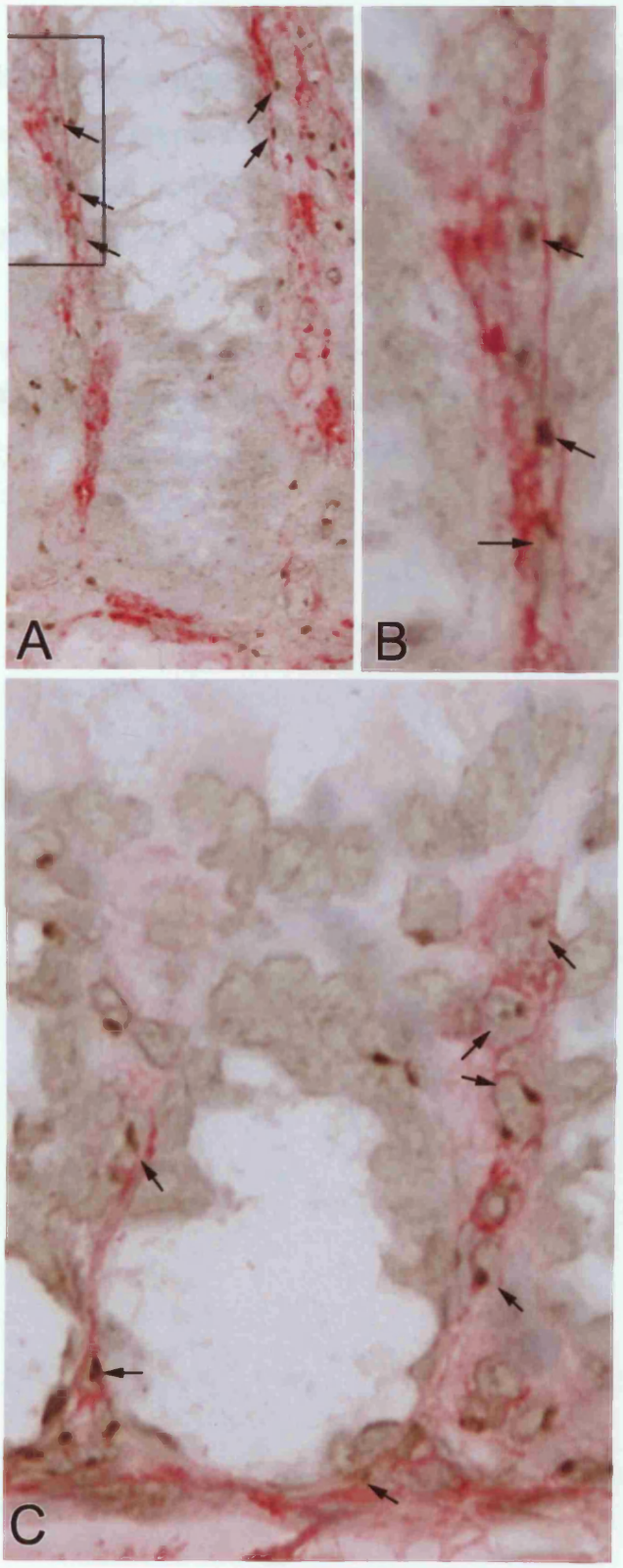

Figure 4.3) BM-derived ISEMFs form cellular columns in the lamina propria.

BM-derived ISEMFs were present as cellular columns spanning from the base of the epithelial crypts to the intestinal lumen, at all timepoints studied in both ethanol controls and in the inflamed and non-inflamed mucosa of TNBS-treated mice. ISEMFs were identified by their Y chromosome expression (dark brown nuclear spot) and SMA immunoreactivity (red cytoplasm). This figure shows columns of BM-derived ISEMFs in the non-inflamed mucosa in ethanol-treated mice (A, and inset B, C; arrows; 6 days post-ethanol).

Figure 4.4

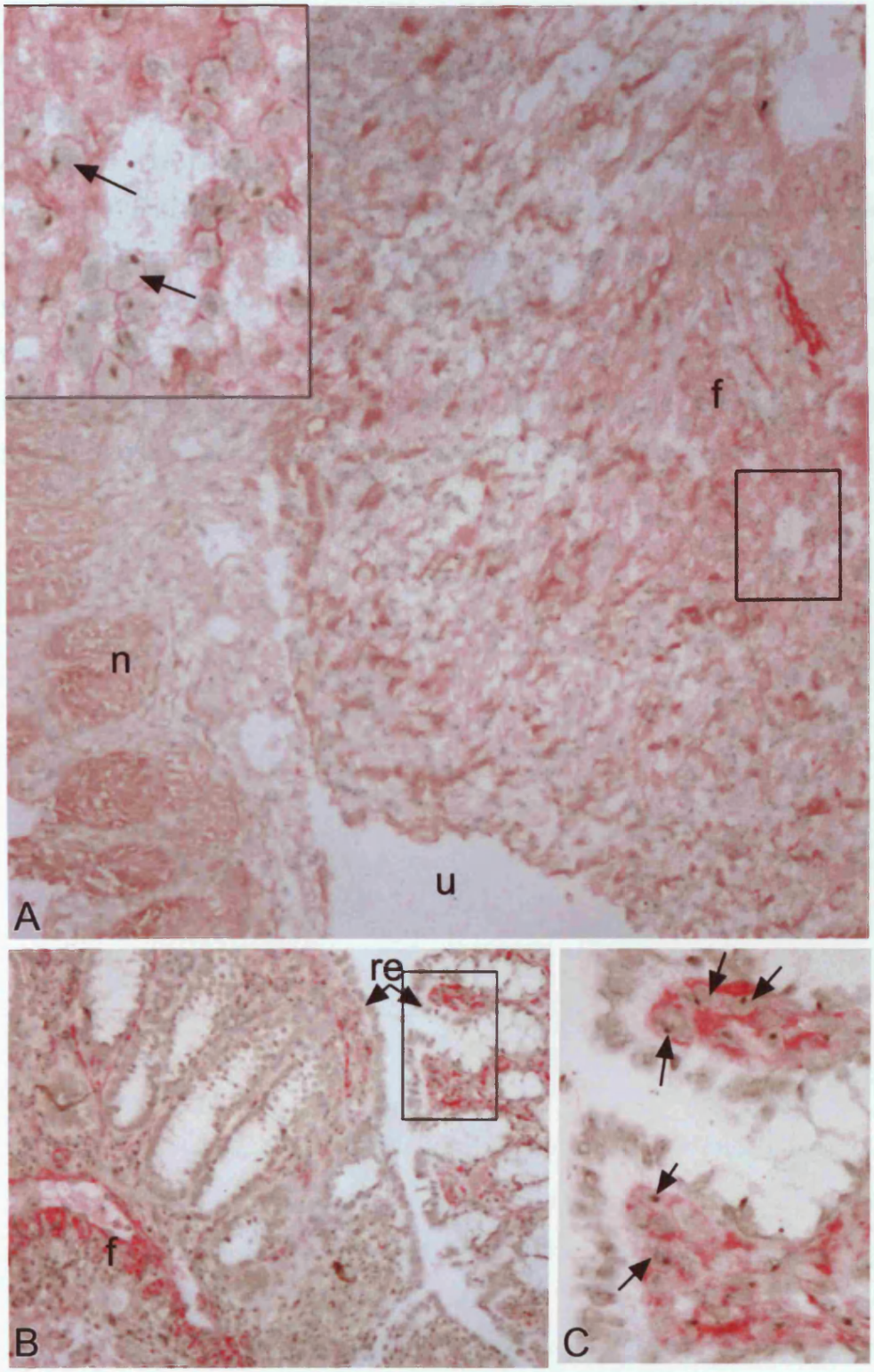

Figure 4.4) BM-derived ISEMFs display an activated phenotype in TNBS colitis.

Activated myofibroblasts in colitis were identified by their location in regions of fibrosis and by their large, flat shape (A – C; arrows; 6 – 8 days post-TNBS). BM-derived cells were present in the lamina propria surrounding regions of mucosal re-epithelialisation, suggesting a potential role for BM-derived cells in mucosal regeneration (B, and high power inset C; arrows; 8 days post-TNBS). n, epithelial cell necrosis and crypt death; u, mucosal ulceration; f, fibrosis; re, re-epithelialisation.

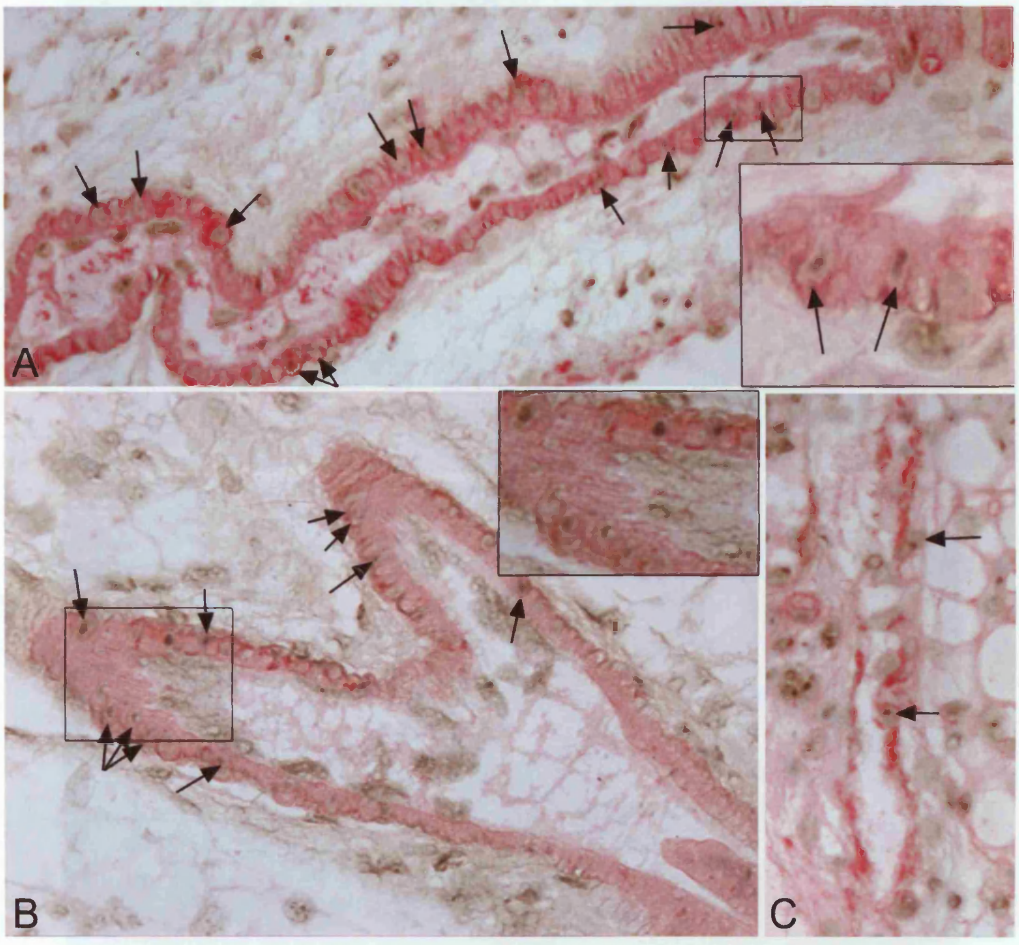

Figure 4.5) BM forms mural cells in blood vessels in TNBS colitis. BM-derived mural cells were identified as smooth muscle-like cells surrounding the blood vessels that were immunoreactive for SMA antigen (red cytoplasm) and possessed a nuclear Y chromosome (dark brown nuclear spot). BM-derived mural cells were frequently interspersed throughout blood vessels in inflamed colons (A and high power inset, B and high power inset, C; arrows; 6 days post-TNBS).

Figure 4.6

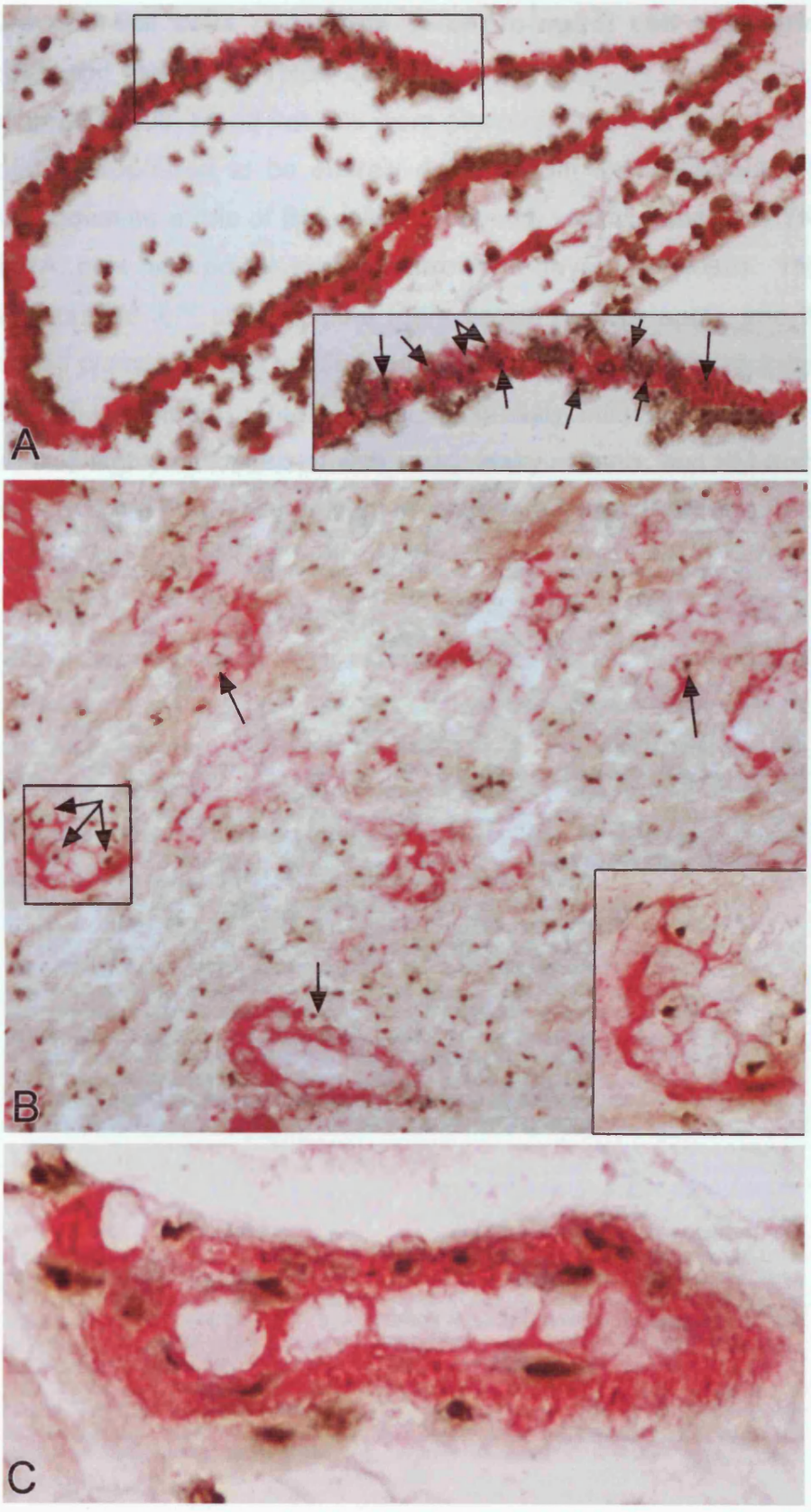

Figure 4.6) BM cells contribute to entire mural cell populations within blood vessels in TNBS colitis.

In inflamed tissue, blood vessels were observed in which the mural cell population appeared to be entirely derived from the transplanted BM cells, suggesting a role of BM cells in postnatal vasculogenesis in TNBS colitis (A, plus high power inset, C; arrows; 6 days post-TNBS). These cells displayed a Y chromosome (dark brown nuclear spot), and their mural cell phenotype was confirmed by their positive immunoreactivity for SMA (red cytoplasm). Interestingly, in grossly inflamed regions, the tissue was highly vascularised with small, leaky vessels, and BM-derived mural cells were frequently present in these structures (B; arrows; 8 days post-TNBS).

Figure 4.7

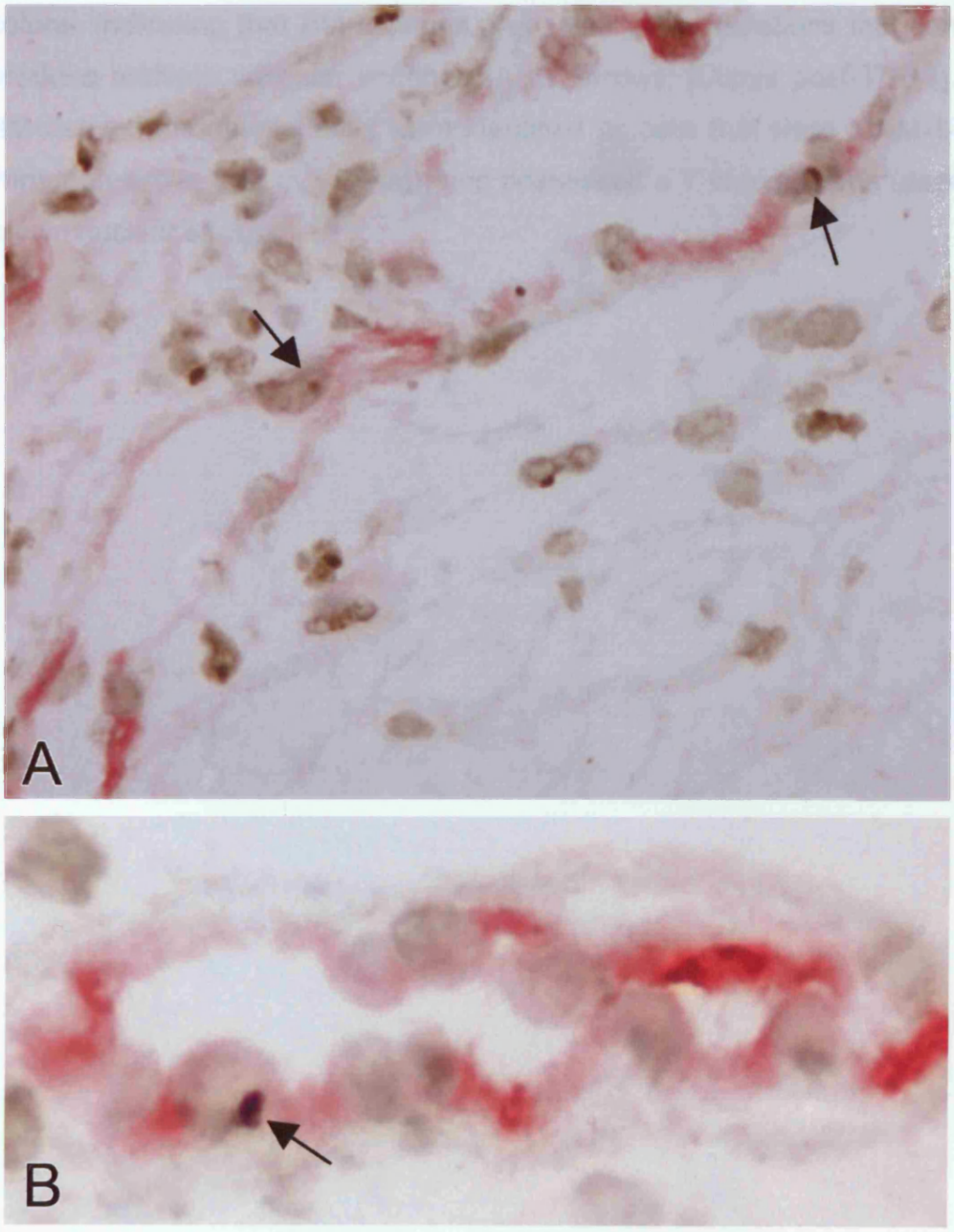

Figure 4.7) BM contributes to endothelial cells in TNBS colitis.
BM-derived endothelial cells were identified in blood vessels in inflamed colons, indicating that BM contains progenitor cell populations that can produce multiple vascular lineages (A, B; arrows; 8 days post-TNBS). BM-derived endothelial cells were identified as cells that were ICAM-1-immunoreactive (red cytoplasm), and possessed a Y chromosome (dark brown nuclear spot).

Figure 4.8

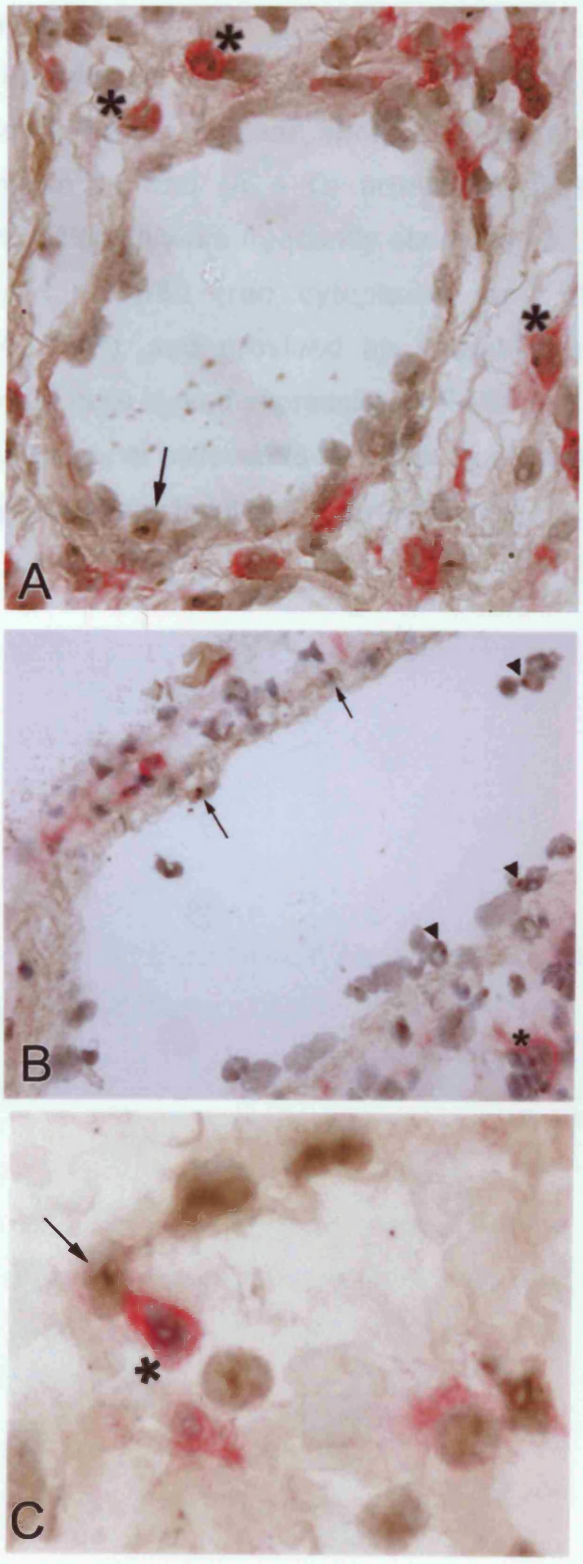

Figure 4.8) Some BM-derived cells in blood vessels do not express the macrophage marker F4/80.

Blood vessels in inflamed colons contained cells that displayed a Y chromosome (dark brown nuclear spot), but did not express the macrophage marker, F4/80 (A – C; arrows; 6 days post-TNBS). Macrophages of BM origin were frequently observed i.e., cells that were immunoreactive for F4/80 (red cytoplasm) and displayed a Y chromosome (A, C; *), and provided an internal control for these experiments. Some cells lacked expression of F4/80 and expressed a Y chromosome, but were not believed to be either mural or endothelial cells due to their morphology and location (B; arrowheads).

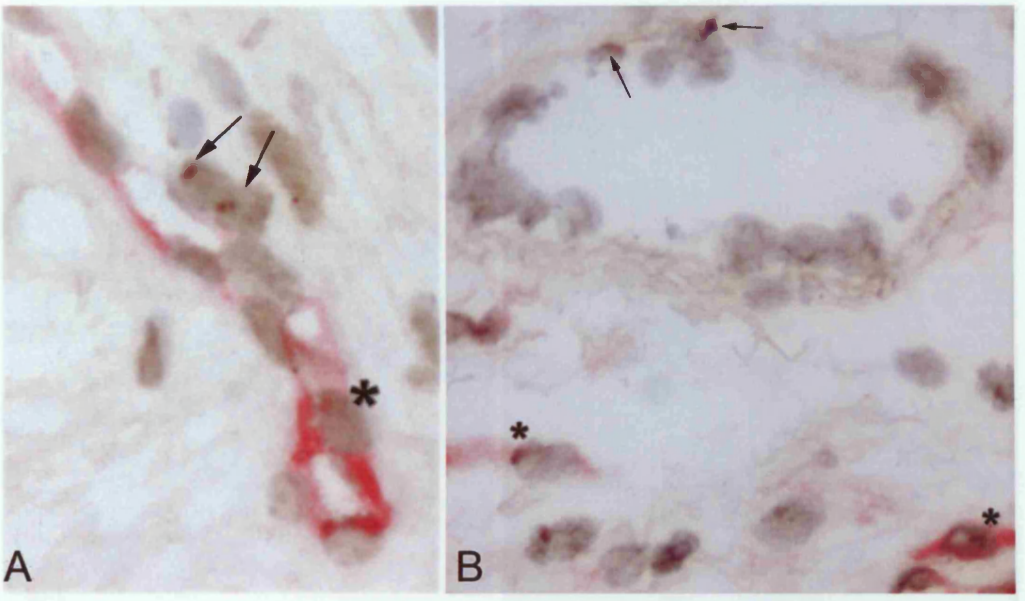

Figure 4.9) Some BM-derived cells in blood vessels do not express the haematopoietic cell marker CD34.

Blood vessels in inflamed colons contained cells that displayed a Y chromosome (dark brown nuclear spot), but did not express the haematopoietic marker, CD34 (A, B; arrows; 6 days post-TNBS). Haematopoietic cells derived from the transplanted BM were frequently observed i.e., cells that were immunoreactive for CD34 (red cytoplasm) and displayed a Y chromosome (A, B; *), and provided an internal control for these experiments.

Figure 4.10

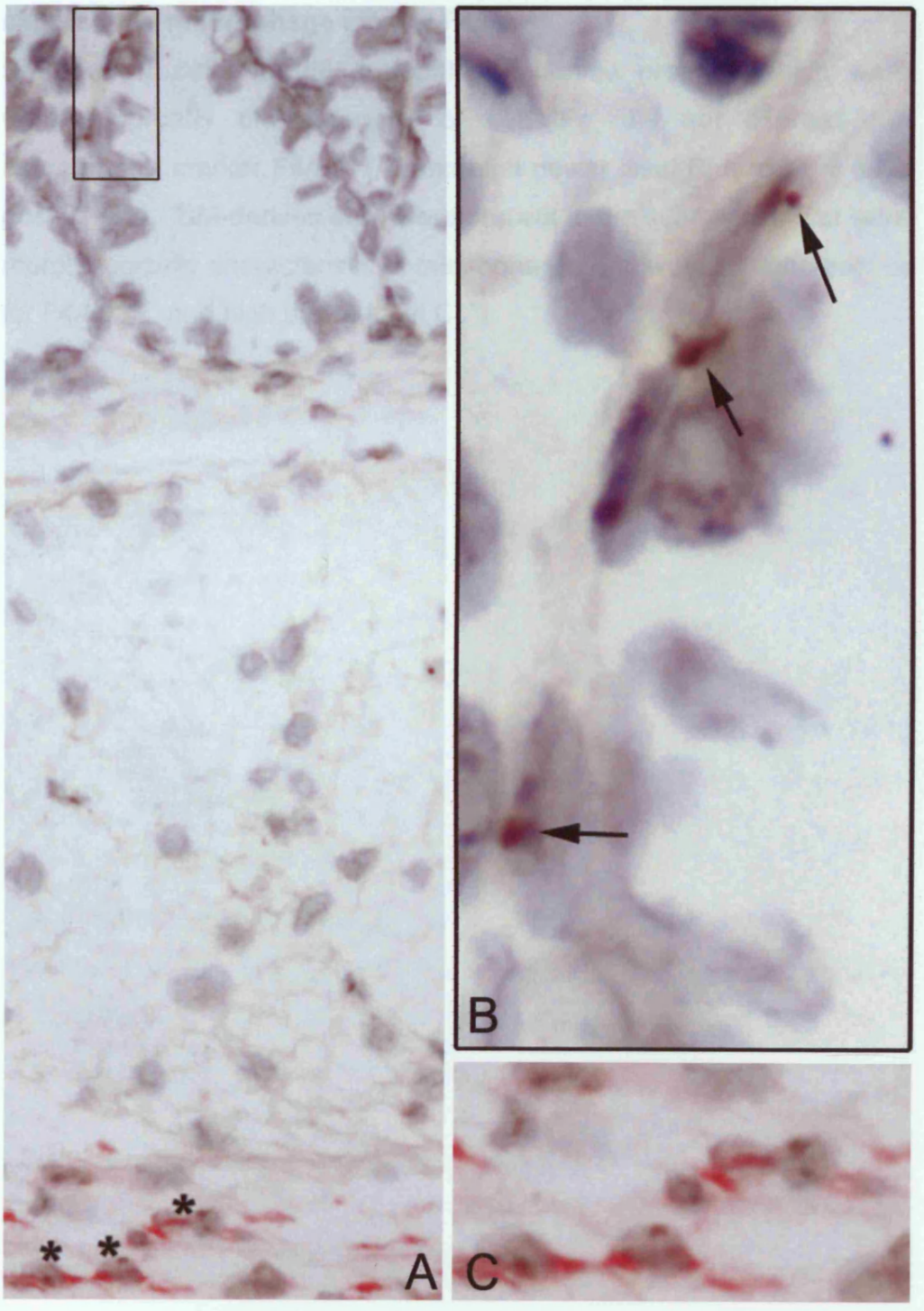

Figure 4.10) Some BM-derived cells in the lamina propria do not express the macrophage marker F4/80.

BM-derived cells in the subepithelial lamina propria, which were morphologically characteristic of ISEMFs, did not express the macrophage marker F4/80 (A, and high power inset B; arrows; 6 days post-TNBS). BM-derived cells were present in the submucosa that were morphologically characteristic of macrophages and were immunoreactive for F4/80 (A, and high power inset C; *).

CHAPTER V

BM-derived keratinocytes in normal and wounded epidermis: evidence of proliferate competence *in vivo* and *in vitro.*

5.1) Introduction

5.1.1) The adult mouse epidermis has two stem cell populations

The stem cells of the adult mouse epidermis reside within two distinct locations, the epidermal proliferating units (EPU) in the basal layer of the IFE, and the bulge region of the hair follicles. The stem cells in the bulge are more numerous than those within EPUs, and can undergo multipotent differentiation to give rise to all the differentiated adult lineages in the hair follicles, sebaceous glands and IFE, whereas the stem cells within EPUs are unipotent and contribute solely to keratinocytes of the IFE (section 1.8).

As stem cells cycle more slowly than their differentiating counterparts, the majority of studies aimed at identification of the epidermal stem cells have utilised the slow cell cycle duration of these cells, and their resultant DNA label-retaining phenotype (section 1.8).

5.1.2) Markers of stem cells in the epidermis

Recent literature has highlighted several molecular markers that display an enriched expression in cells within the postulated epidermal stem cell niches, including the β1- and α6-integrin subunits (section 1.9.1) and the keratin proteins, K15 and K19 (section 1.9.2). CD34, previously believed to be restricted to early haematopoietic progenitor cells, was recently

shown to be co-expressed in K15-, α6 integrin-positive keratinocytes in the follicular bulge (section 1.9.2).

5.1.3) BM cells contribute to adult epidermal lineages

It is now well validated that adult BM cells are not all lineage-restricted and can engraft within foreign tissues to produce functional non-haematopoietic cell types (section 1.13). Recent studies have shown that BM-derived cells of epithelial phenotype can be found in the epidermis (section 1.13.3; (Badiavas et al., 2003; Borue et al., 2004; Kataoka et al., 2003; Korbling et al., 2002; Krause et al., 2001)), although the functional capacity of these cells is poorly understood and many questions remain to be answered. For example, do BM cells contribute to epidermal stem cell niches? Do BM-derived epidermal cells have the potential to proliferate and produce differentiated progeny? Is BM cell engraftment enhanced in cutaneous wound healing? And finally, since BM-derived cells appear to engraft in various tissues by either spontaneous fusion or by *de novo* cell generation, what is the mechanism of engraftment of BM cells in the epidermis?

5.2) Aim

The following section of this project is an investigation into the contribution of transplanted BM cells to the cells of the normal and wounded mouse epidermis. Previous studies have shown that transplanted BM cells can engraft within the skin and assume a keratinocyte phenotype, and we therefore aimed to investigate whether these BM-derived cells; (1) are capable of engraftment into the keratinocyte stem cell niches, (2) have the potential to proliferate and form colonies *in vivo* and *in vitro*, (3) contribute to cutaneous wound repair and, (4) form epidermal lineages by *de novo* generation of new cells, or by fusion with an indigenous keratinocyte to form a heterokaryon.

5.3) Methods

Female mice were lethally irradiated and rescued by a whole BM transplant from GFP-positive male donors (section 2.1). Six weeks post-transplant, the dorsal skin of recipient mice was wounded by a 3 mm punch biopsy, and mice were killed at 4, 7 or 30 days post-wounding. Mice were injected with BrdUrd one-hour prior to sacrifice, to label cells in S-phase.

For the spontaneous fusion study, male mice were given a BM transplant from GFP-positive male donors, which permitted identification of the donor-derived keratinocytes by their co-expression of GFP and K14. *In situ* hybridisation was then performed to detect Y chromosome number within the BM-derived keratinocytes, to determine whether fusion of a male donor cell with an indigenous male keratinocyte had occurred.

Following BM transplant and cutaneous wounding, the skin was harvested and processed in a number of different ways depending on the method of analysis performed i.e., normal and wounded epidermis was fixed in NBF and paraffin-embedded for immunohistochemical and *in situ* hybridisation analyses of tissue sections, tissue wholemounts were prepared from the tail epidermis for immunofluorescence and confocal analyses, epidermal keratinocytes were isolated and grown in culture for immunofluorescence, and DNA was extracted from keratinocyte colonies for PCR.

The materials and methods used in this series of experiments are described in full in sections 2.2.3, 2.3 - 2.6.

5.4) Results

5.4.1) BM-derived cells engraft within the epidermis and form keratinocyte-like cells

Six weeks after BM transplant, GFP-positive cells were frequently observed throughout the epidermis; in the IFE, the hair follicles including the bulge region, and in the sebaceous glands. Many of these donor-derived cells were characteristic of keratinocytes, as shown by their rounded, vesicular nuclei, abundant non-dendritic cytoplasm and epithelial cell-like morphology (Figure 5.1 – 5.3). Immunohistochemical controls confirmed a lack of non-specific binding by the GFP antibody (Figure 5.4).

BM-derived cells were also present in the dermis, and were believed to be circulating inflammatory lineages. Some cells of BM origin in the IFE displayed a dendritic phenotype and were likely to be epidermal Langerhans cells (Figure 5.2B), supported by evidence that BM contains Langerhans cell progenitors (Ogata et al., 1999; Reid et al., 1990). It is unlikely that these dendritic BM-derived cells are Merkel cells, as these cells are extremely rare in the back skin of mice (Moll et al., 1996).

5.4.2) BM-derived cells express markers of basal keratinocyte differentiation

The keratinocyte phenotype of the BM-derived cells in paraffin sections of non-wounded epidermis was established either by double immunofluorescence staining for both GFP and K14 (Figure 5.5), or by combining *in situ* hybridisation for the Y chromosome with immunohistochemistry for K14. The numbers of BM-derived keratinocytes were calculated in the hair follicles and in the IFE of non-wounded skin at 6 weeks post-transplant, which revealed that 7.2% and

7.7% of keratinocytes were of donor origin within each respective epidermal region (Table 5.1).

5.4.3) Effects of wounding on BM engraftment into the epidermis

Immunohistochemical detection of GFP revealed that BM-derived cells were abundant throughout the wounded epidermis and the dermis (Figure 5.6A). The keratinocyte phenotype of the BM-derived cells in the wounded epidermis was established either by double immunofluorescence staining for both GFP and K14 (Figure 5.6B), or by combining *in situ* hybridisation for the Y chromosome with immunohistochemistry for K14 (Figure 5.7). BM-derived keratinocytes were commonly present as rows of adjacent cells in regions of re-epithelialisation (Figure 5.6B). At 4 days post injury, the fraction of BM-derived basal keratinocytes in the hyperproliferative region of the epidermis was 11.5%, a significant increase when compared 7.2% of keratinocytes in the surrounding non-wounded IFE ($p = 0.0028$; Table 5.1).

Double immunohistochemical staining for GFP with the macrophage marker, F4/80 (Figure 5.8A), or the neutrophil marker, Ly6G (Figure 5.8B-D) confirmed that many of the dermal cells in the wound bed were circulating inflammatory lineages, as expected.

5.4.4) BM-derived cells are present in the epidermal stem cell regions and can express CD34

Donor-derived cells were detected by *in situ* hybridisation for the Y chromosome, and this was combined with immunohistochemistry for the putative epidermal stem cell marker CD34. CD34-immunoreactive cells were concentrated in the region of the hair follicles just beneath the sebaceous glands. We observed BM-derived cells in this region that also expressed CD34 (Figure 5.9).

Rows of GFP-positive cells were observed in structures resembling EPUs in the regenerating IFE at 4 days post-wounding (Figure 5.10A). Interestingly, the cornified layer of dead cells shed from the surface of the epidermis above the GFP-positive EPU was also GFP-positive, whereas cells shed from regions of non-GFP-positive epidermis were not.

5.4.5) Epidermal wholemounts provide a 3-dimensional perspective of BM cell engraftment into the epidermis

We employed a novel method to examine BM-derived cell engraftment in the epidermis using wholemount preparations of tail skin from wild type mice transplanted with GFP-positive male BM. Confocal analyses of the wholemounts provided a three-dimensional perspective of the epidermis and a greater insight into the location of the engrafted BM-derived cells. Double immunofluorescence-labelling for GFP and K14 identified basal keratinocytes of donor origin. Our results confirmed our observations in paraffin sections, that BM-derived keratinocytes were frequently located in the hair follicle, just beneath the sebaceous gland (Figure 5.11).

5.4.6) BM-derived keratinocytes can incorporate BrdUrd

Transplanted mice were given a single pulse of BrdUrd one-hour prior to sacrifice. Approximately 4 - 6% of the total epidermal keratinocyte population, of both host and donor origin, incorporated BrdUrd following this pulse. Cells that were triple immunofluorescence-labelled for GFP, K14 and BrdUrd were observed in both the non-wounded and regenerating IFE, thus demonstrating that BM-derived keratinocytes were capable of proliferation (Figure 5.12).

In addition, large rows of GFP-positive cells were observed in the ORS of hair follicles 30 days post injury (Figure 5.10B), although these were not observed in the hair follicles of non-wounded skin, indicating the importance of wounding and that BM-derived cells are maintained in the epidermis when the tissue has regenerated.

5.4.7) BM-derived keratinocytes undergo clonal expansion *in vitro*

Keratinocyte cultures were established from single cells isolated from the dorsal skin of transplanted mice, and were maintained in normal calcium-containing medium and grown on a feeder layer of fibroblasts. Cultures were double-fluorescence immunostained for GFP and K14. We observed 6 out of 600 colonies that expressed both GFP and K14 (Figure 5.13), suggesting these colonies were each derived from a single BM-derived keratinocyte.

Furthermore, PCR analysis of both GFP and the Y chromosome of DNA extracted from the GFP-positive; K14-positive cell colonies, confirmed that the keratinocytes in these cultures derived from transplanted mice were of BM origin (Figure 5.14).

5.4.8) Spontaneous fusion is not detectable in BM-derived keratinocytes

Paraffin sections of epidermis from male wild-type hosts transplanted with male GFP-positive BM were analysed for the combined expression of GFP, K14, and the Y chromosome (5 mice, 200 cells from each mouse). Paraffin sections were 10 μm thick to ensure incorporation of entire nuclei. On no occasion were multiple Y chromosomes observed within a keratinocyte. Moreover, GFP-positive, K14-positive cells that contained a single Y chromosome were seen (Figure 5.15), implying that transplanted BM cells can form keratinocytes without prior fusion with an indigenous cell.

5.5) Discussion

My observations, that BM-derived cells can not only engraft with high frequency in known epidermal stem cell niches, but also show proliferative potential *in vivo*, and colony formation *in vitro*, were unexpected and call for a reconsideration of the possible source of adult stem cells within the epidermis.

5.5.1) BM-derived cells engraft within the epidermis and contribute to the stem cell niche

While much has been learned about the signals that regulate self-renewal and lineage commitment choices of epidermal stem cells (Fuchs and Raghavan, 2002; Niemann and Watt, 2002), one question that has not been addressed is the origin of adult epidermal stem cells. My observations of BM-derived keratinocytes in the hair follicles, IFE and sebaceous glands indicate that BM cells can engraft within the adult epidermis and undergo differentiation to form specialised epidermal cell lineages.

Engrafted BM-derived keratinocytes were frequently observed in epidermal stem cell niches i.e., the follicular bulge region and EPU-like structures in the IFE. Moreover, BM-derived cells in the bulge could express the putative stem cell marker CD34, raising the possibility that these cells may participate in epidermal homeostasis. BM-derived EPU-like structures appeared morphologically typical of indigenous EPUs, and were composed of approximately 10 cells in the basal epidermis. Furthermore, BM-derived EPU-like structures also appeared to be functioning normally, as the stratum corneum immediately above these structures was also GFP-positive, although surrounding cells in the stratum corneum did not express GFP. This is highly suggestive that BM-derived cells within an EPU show stem cell-like behaviour by producing

differentiated progeny that migrate upward through each layer of the epidermis until they are shed from the skin surface.

5.5.2) BM-derived keratinocytes are significantly increased in regenerating epidermis

Tissue injury has been proposed to enhance engraftment of BM cells in many organs (section 1.13). To investigate whether this applies to the mouse epidermis, full thickness cutaneous wounds were applied to dorsal skin of transplanted mice, and wounded tissue was harvested at 4, 7 or 30 days post-injury for histological analysis of BM cell recruitment. Cutaneous wound healing involves the temporal and spatial regulation of various cell types including infiltrating immune cells such as neutrophils, macrophages and fibroblasts, in addition to the processes of re-dermalisation and re-epithelialisation. The latter involves hyperproliferation and migration of epidermal keratinocytes from the edge of the wound to cover the neodermis.

The frequency of BM-derived cells was increased significantly in wounded skin (11.6%) compared with non-wounded skin (7.2%), and I commonly observed colonies of BM-derived keratinocytes in the wounded epidermis. These data imply a functional role for BM cells in epidermal regeneration, although it is not clear if BM engraftment is increased after wounding, or if BM-derived keratinocytes already established within the epidermis increase their proliferation following damage, or indeed if both scenarios occur.

Interestingly, I observed rows of BM-derived cells in the ORS of the hair follicles at 30 days post-wounding when the epidermis had regenerated, which demonstrates the sustained expression of BM-derived cells in the epidermis after injury, and also suggests that BM-derived keratinocytes can proliferate to produce daughter cells that can leave the stem cell niche and form differentiated cells in the hair follicle.

5.5.3) BM-derived keratinocytes proliferate *in vivo* and can form colonies *in vitro*

BM-derived keratinocytes were observed in the non-wounded and regenerating epidermis that had incorporated a BrdUrd label and were therefore undergoing proliferation *in vivo*. This is the first observation of BM-derived keratinocytes in S-phase, and these data provide strong evidence for a functional role of BM-derived keratinocytes within the epidermis i.e., in the maintenance of the continual turnover of differentiated cells.

Furthermore, BM-derived keratinocytes formed colonies when cultured *in vitro*, thereby substantiating, for the first time, the capacity of BM cells to undergo clonal expansion and produce differentiated keratinocyte progeny.

5.5.4) BM-derived keratinocytes appear to form with no evidence of fusion

The regulatory mechanisms of BM cell differentiation, and the role of BM in epidermal regeneration are not known. Recent literature suggests that in some tissues, manifestation of a differentiated phenotype by ingressing adult stem cells is a result of cell fusion with a pre-existing adult cell to form a heterokaryon, although evidence also exists to show the *de novo* generation of cells from BM with no evidence of fusion (section 1.14). My results advocate the hypothesis that BM cells can form differentiated adult lineages in the epidermis, and indicate that BM-derived epidermal cells are unlikely to be a product of cell fusion. My data correlates with very recently published work suggesting the same (Harris et al., 2004). It is possible that BM-derived epidermal cells shed super-numerary nuclei, although this is deemed unlikely because heterokaryons appear stable for long periods of time (Weimann et al., 2003b).

5.6) Conclusion

I believe that this study establishes that BM cells engraft into the epidermal stem cell niche and participate in tissue regeneration, and can form epidermal cells without fusion. I have also demonstrated for the first time, that BM-derived keratinocytes are capable of initiating cell proliferation *in vivo* and can divide to produce differentiated keratinocyte colonies *in vitro*.

Although beyond the scope of this report, these data raise some important questions regarding BM-derived stem cells in the epidermis. For example, how do BM cells 'home' to the bulge and epidermis? What are the stimuli to enhance BM-derived cell engraftment within wounded skin? Will a clearer understanding of the mechanisms of BM cell plasticity highlight the potential roles of BM-derived cells in the epidermis? The underlying principal, that transplanted adult BM cells assume the phenotype of their host tissue with an emphasis in diseased or damaged tissue, cannot be overlooked. Moreover, given that the results raise the possibility that BM may be a source of proliferating epidermal stem cells, the use of BM in the treatment of human skin disease becomes an important option. Further analyses of the mechanisms a cell employs to acquire a differentiated epidermal cell phenotype will be required prior to clinical application.

Table 5.1) Donor-derived keratinocytes in normal and wounded epidermis of BM transplanted mice[a].

Y chromosome-positive keratinocytes/ total keratinocytes (%)			
Mouse ID	Normal IFE	Hair Follicle	Wound
10410/01	15/266 (5.6%)	39/398 (9.8%)	101/1035 (9.8%)
10411/01	17/260 (6.5%)	24/287 (8.3%)	101/845 (11.9%)
10407/01	62/738 (8.4%)	65/824 (7.9%)	114/840 (13.6%)
3210/02	18/361 (4.9%)	39/492 (7.9%)	49/369 (13.2%)
5425/02	57/614 (9.2%)	33/705 (4.6%)	N/D
10406/01	36/413 (8.7%)	25/456 (5.4%)	37/417 (8.8%)
X ± SEM[b]	**7.2 ± 0.7**	**7.3 ± 0.8**	**11.5 ± 0.9[c]**

[a] For staining protocol please refer to the Materials and Methods section (Chapter II).

[b] Mean and standard error of the mean (SEM) percentage of Y-positive keratinocytes.

[c] Statistically significant increase in Y chromosome-positive keratinocytes in wounded compared to non-wounded epidermis ($p = 0.0028$; one-tailed t-test).

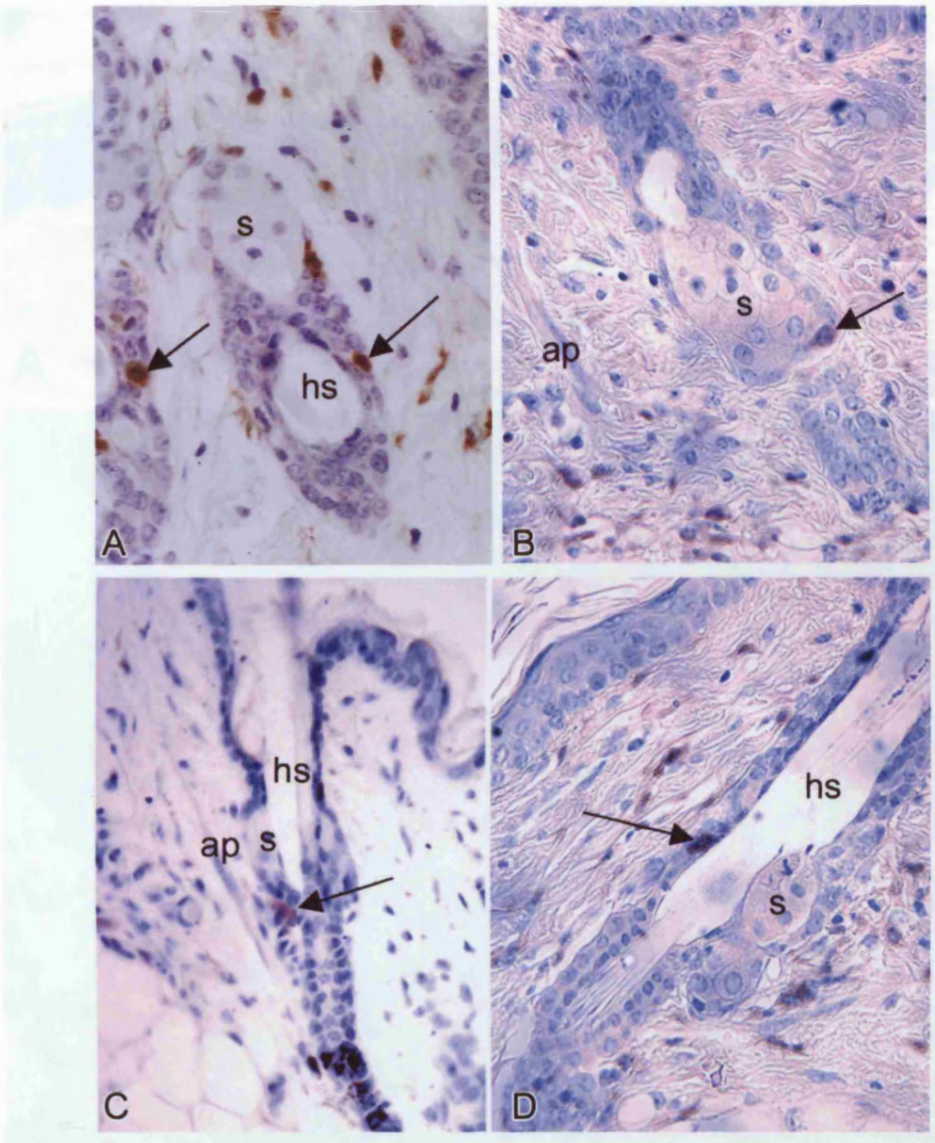

Figure 5.1) BM-derived cells engraft into the hair follicles in non-wounded epidermis.

BM-derived cells were detected by GFP immunoreactivity (dark brown stain) and were frequently identified within hair follicles, and also in the region of the bulge (A – D; arrows). These cells were morphologically characteristic of keratinocytes. The bulge is identified as the lowest point of the upper, permanent portion of the follicle, beneath the sebaceous glands and directly below the point of insertion of the arrector pili muscle. hs, hair shaft; s, sebaceous gland; ap, arrector pili muscle.

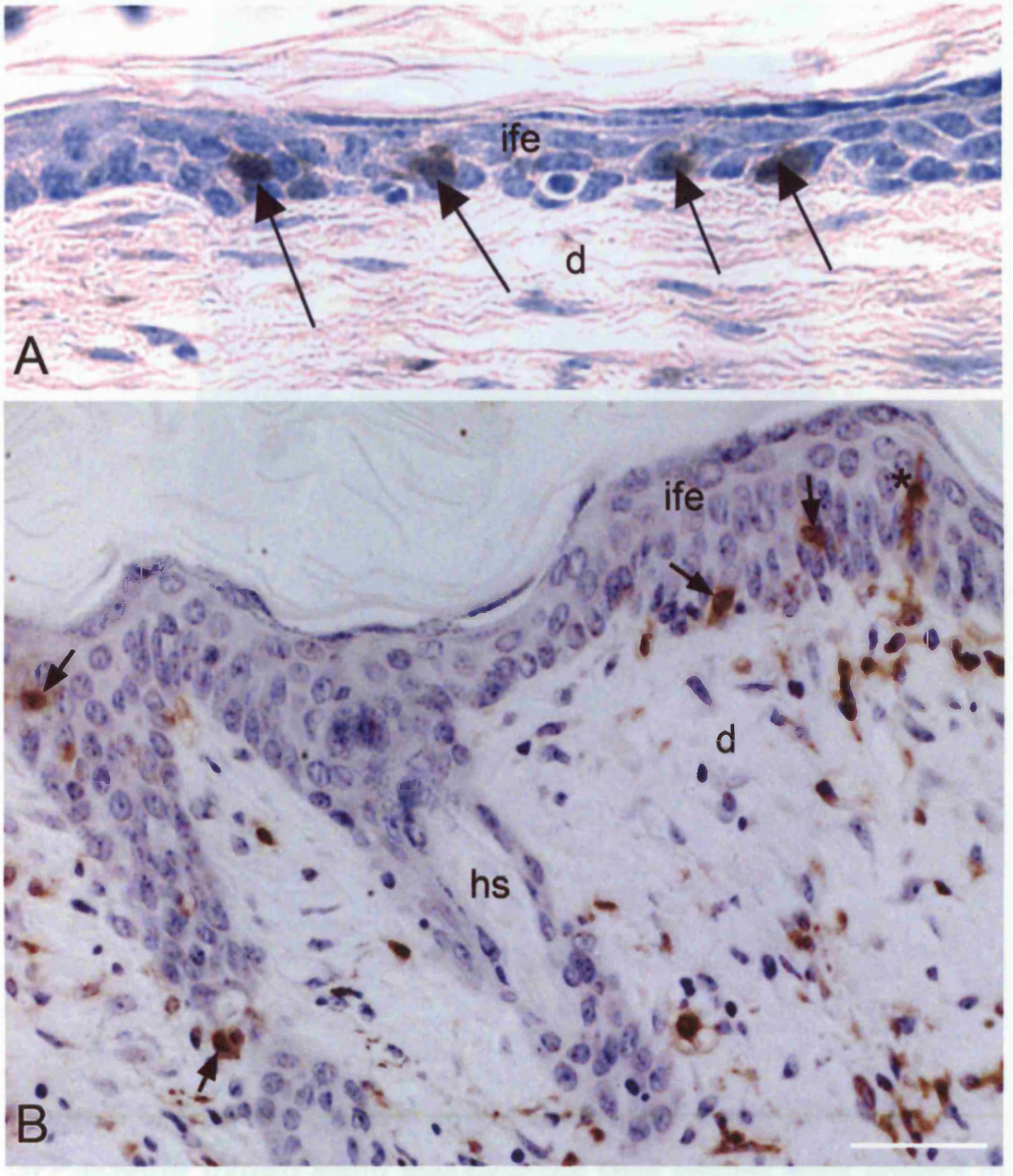

Figure 5.2) BM-derived cells engraft within the non-wounded IFE. GFP-immunoreactive, BM-derived cells (brown stain), morphologically characteristic of keratinocytes, were observed throughout the interfollicular epidermis (A, B; arrows). GFP-positive cells were observed in the epidermis that displayed a dendritic morphology, possibly Langerhans cells (B; *). d, dermis; hs, hair shaft; ife, interfollicular epidermis. Scale bar = 50μm.

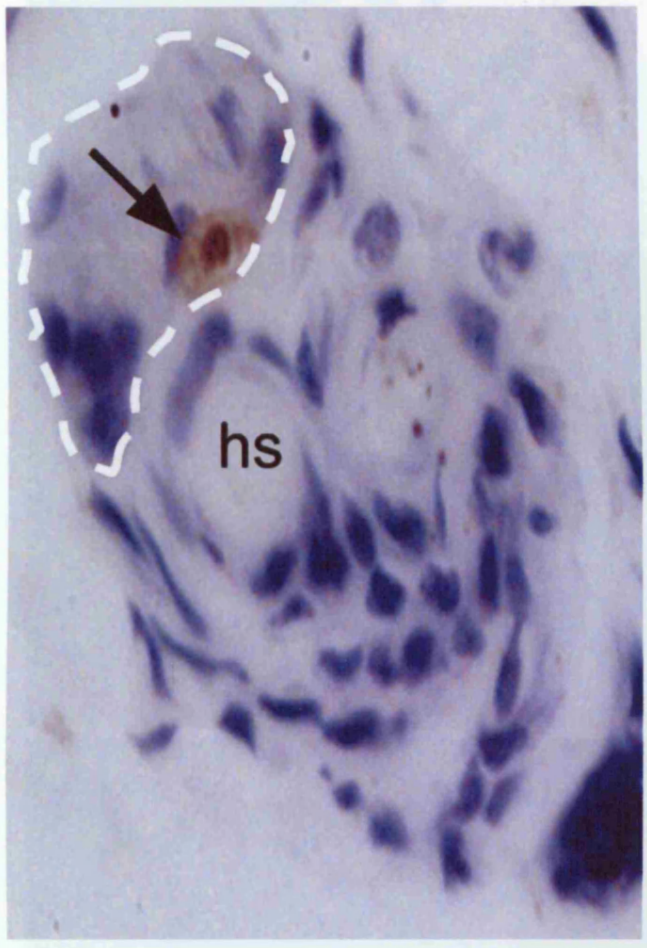

Figure 5.3) Transplanted BM cells can form sebocyte-like cells.
GFP-positive (brown stain) sebocyte-like cells (arrow) were present in sebaceous glands, outlined in this figure by a white dashed line in a cross-section of a hair follicle in non-wounded mouse epidermis 6 weeks after transplant of male GFP-positive whole BM cells. hs, hair shaft.

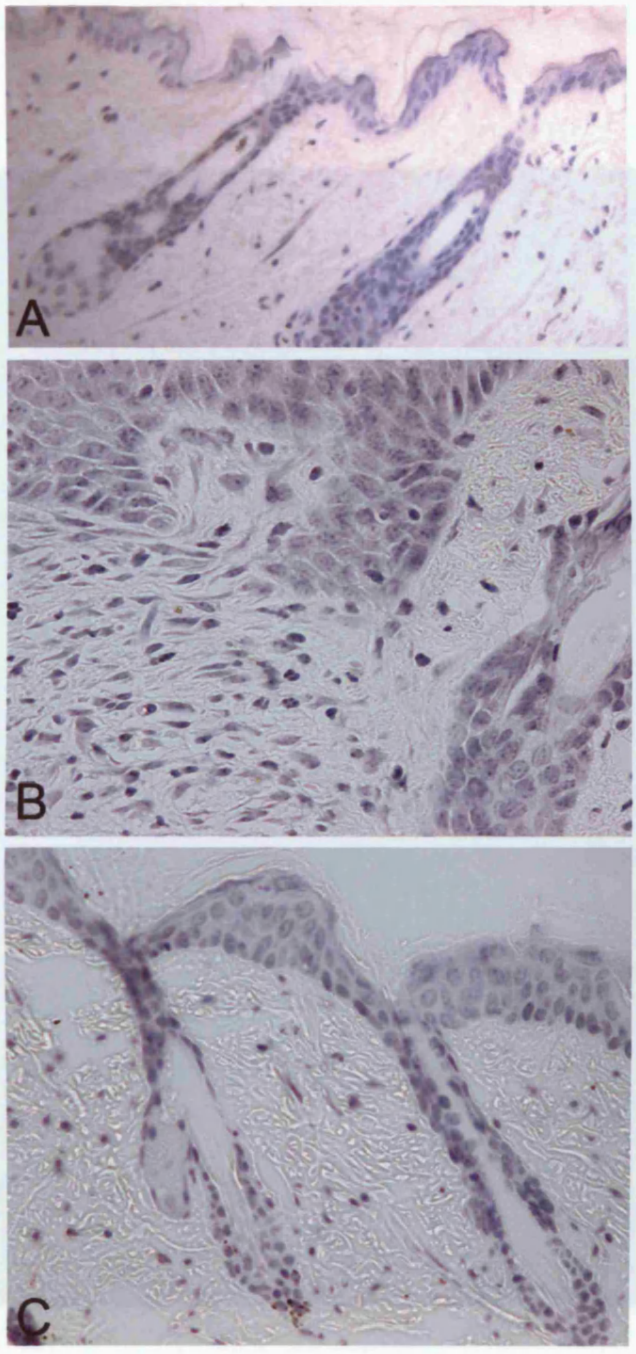

Figure 5.4) Immunohistochemical controls.

Lack of non-specific binding, which would be indicated by a brown stain, in epidermis from GFP transgenic mice probed with isotype-matched IgG control (A). Interfollicular epidermis and hair follicles in epidermis from wild-type mice incubated with the anti-GFP antibody also show lack of non-specific binding (B, C).

Figure 5.5

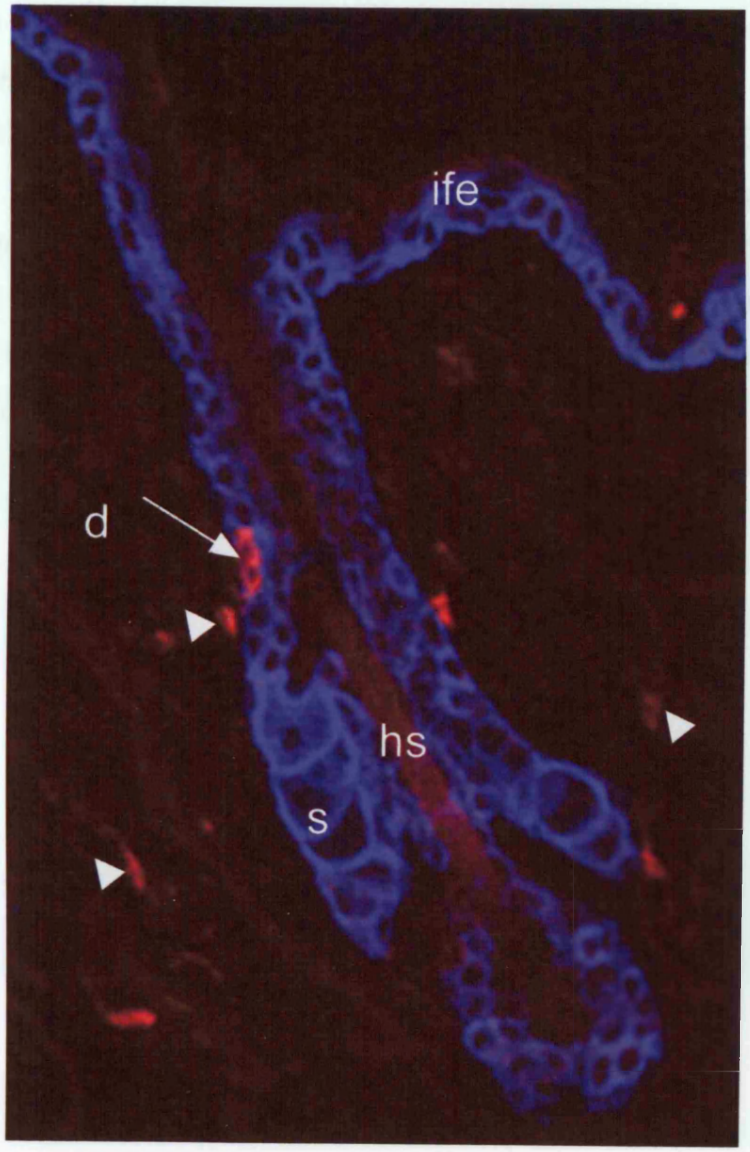

Figure 5.5) BM-derived keratinocytes were present in hair follicles in non-wounded epidermis.

Confocal image of a paraffin section of non-wounded female mouse epidermis at 6 weeks post-transplant, showing BM-derived keratinocyte cells. The section was labelled for both K14 (Cy5 label, blue) and GFP (Cy3 label, red), and cells that co-expressed both K14 and GFP were observed as pink. Multiple, adjacent BM-derived keratinocytes were often observed (arrow). GFP-positive cells were present in the dermis, as expected, that did not express K14 and therefore displayed a red signal and not a pink signal (arrowheads). d, dermis; ife, interfollicular epidermis; hs, hair shaft; s, sebaceous gland.

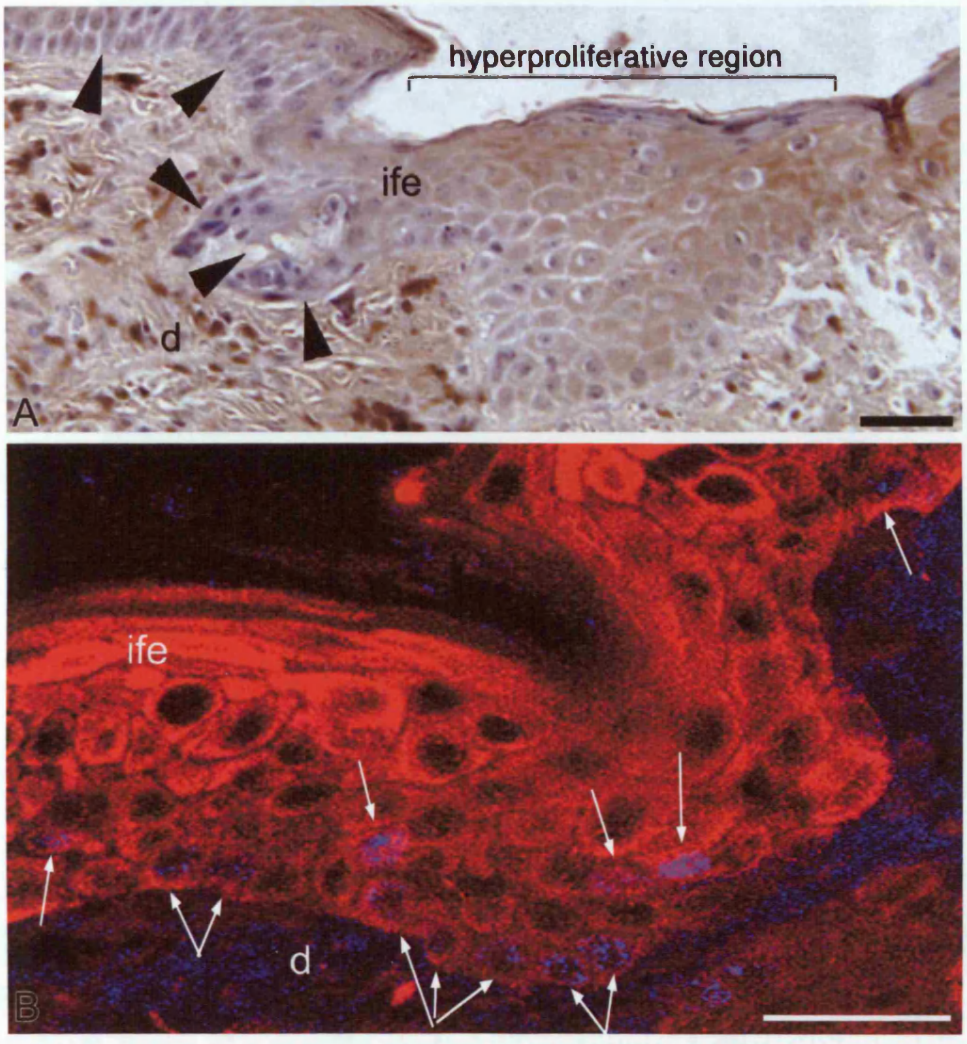

Figure 5.6) BM-derived epidermal cells express K14, and their engraftment is enhanced in wounded epidermis.

An abundance of GFP-positive BM-derived cells (brown signal) were detected in the hyperproliferative region of the regenerating epidermis (A). Most of the keratinocytes just outside this region did not express GFP (A; arrowheads; 4 days post-wounding). BM-derived keratinocyte engraftment increased in response to wounding, shown by multiple adjacent GFP- (Cy5 label, blue), and K14- (Cy3 label, red) doubly positive cells (B; arrows; 4 days post-wounding). ife = interfollicular epidermis, d = dermis. Scale bars = 25μm.

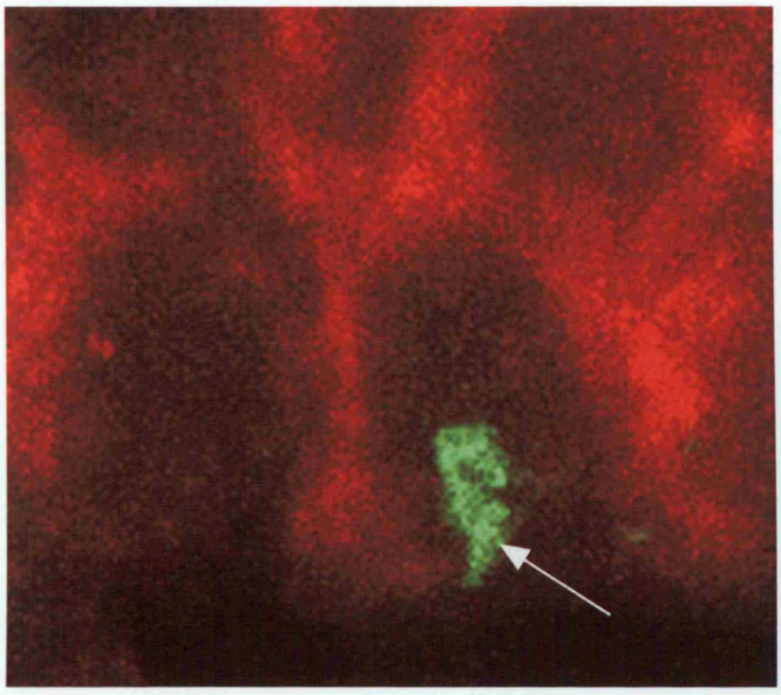

Figure 5.7) High power view of a BM-derived keratinocyte in the regenerating epidermis.

Confocal image of a paraffin section of epidermis, 4 days post-wounding, when re-epidermalisation is taking place. Arrow points to a Y chromosome in the cell nucleus, detected by a FITC-conjugated probe (green), confirming the donor origin of this cell. The keratinocyte phenotype of the cell is demonstrated by positive immunoreactivity for K14 (red cytoplasmic stain).

C

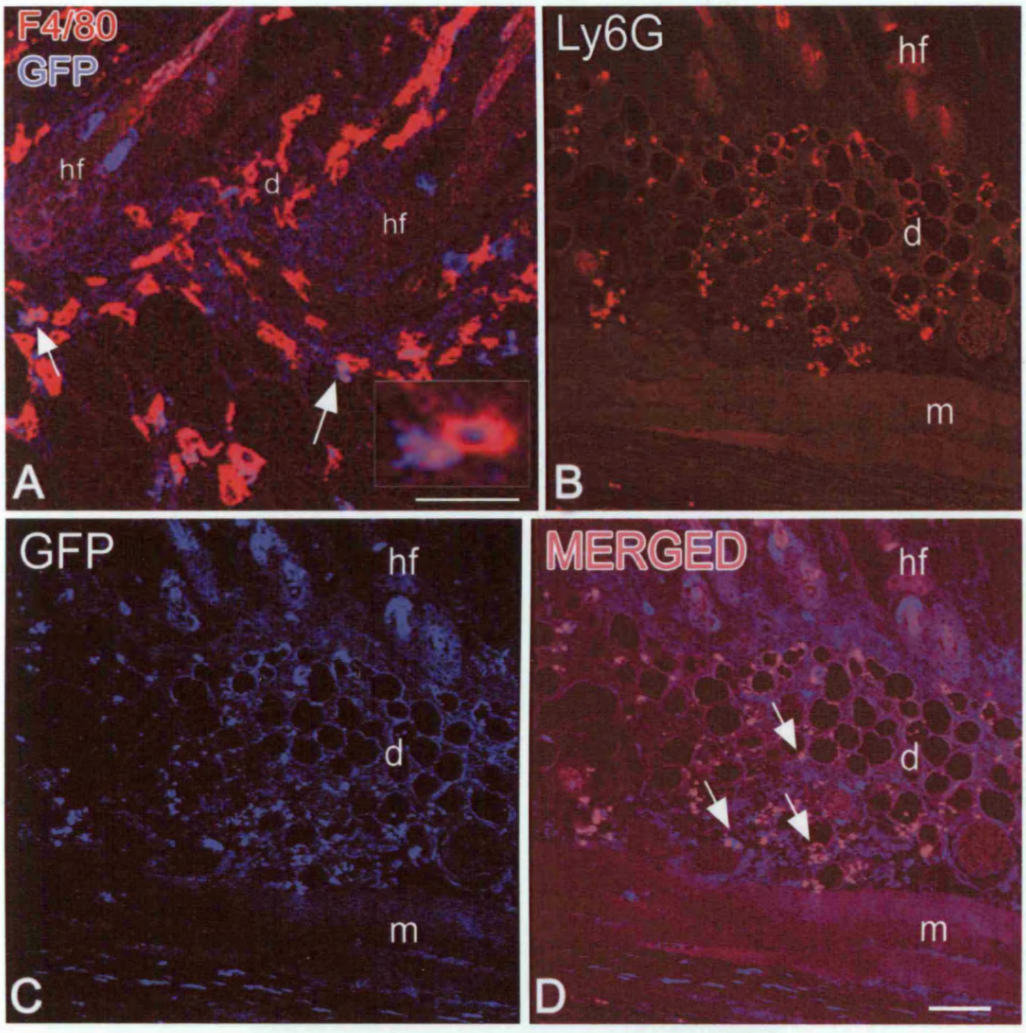

Figure 5.8) Inflammatory cell lineages in the dermis are BM-derived. GFP-positive cells (Cy5 label, blue) that expressed the macrophage marker, F4/80 (Cy3 label, red), were identified by a pink signal (A, and inset; e.g., arrows). Many cells in the dermis co-express the neutrophil marker, Ly6G (B; Cy3 label, red), and GFP antigen (C; Cy5 label, blue), merge of B and C (D; e.g., arrows). hf, hair follicle; d, dermis; m, muscle. Scale bars = 50μm.

Figure 5.9

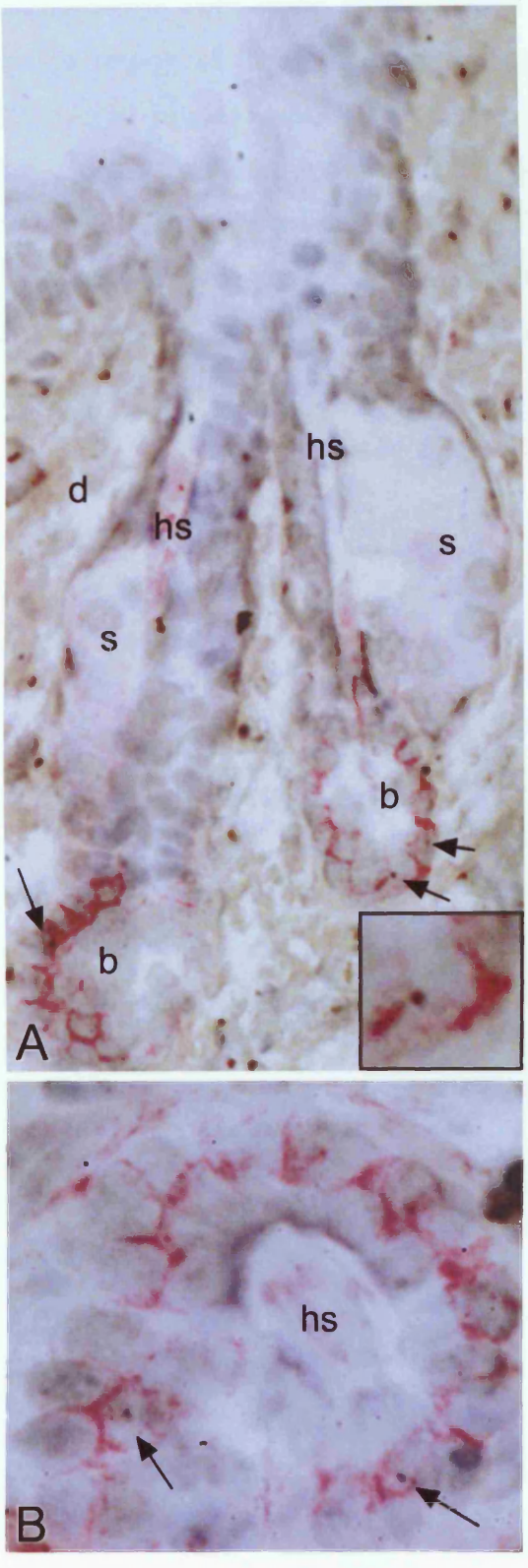

Figure 5.9) BM-derived cells are located in the bulge region of the hair follicles and can express CD34.

In female mice transplanted with male GFP-positive BM, cells were observed in the bulge region of the hair follicles that expressed a Y chromosome (dark brown nuclear spot) and CD34 (red cytoplasm), suggesting that BM-derived cells are located in the putative stem cell zones and express an epidermal stem cell marker. This figure shows BM-derived, CD34-immunoreactive cells in a longitudinal section (A), and in a cross section (B) of hair follicles. hs, hair shaft; d, dermis; s, sebaceous gland; b, bulge region.

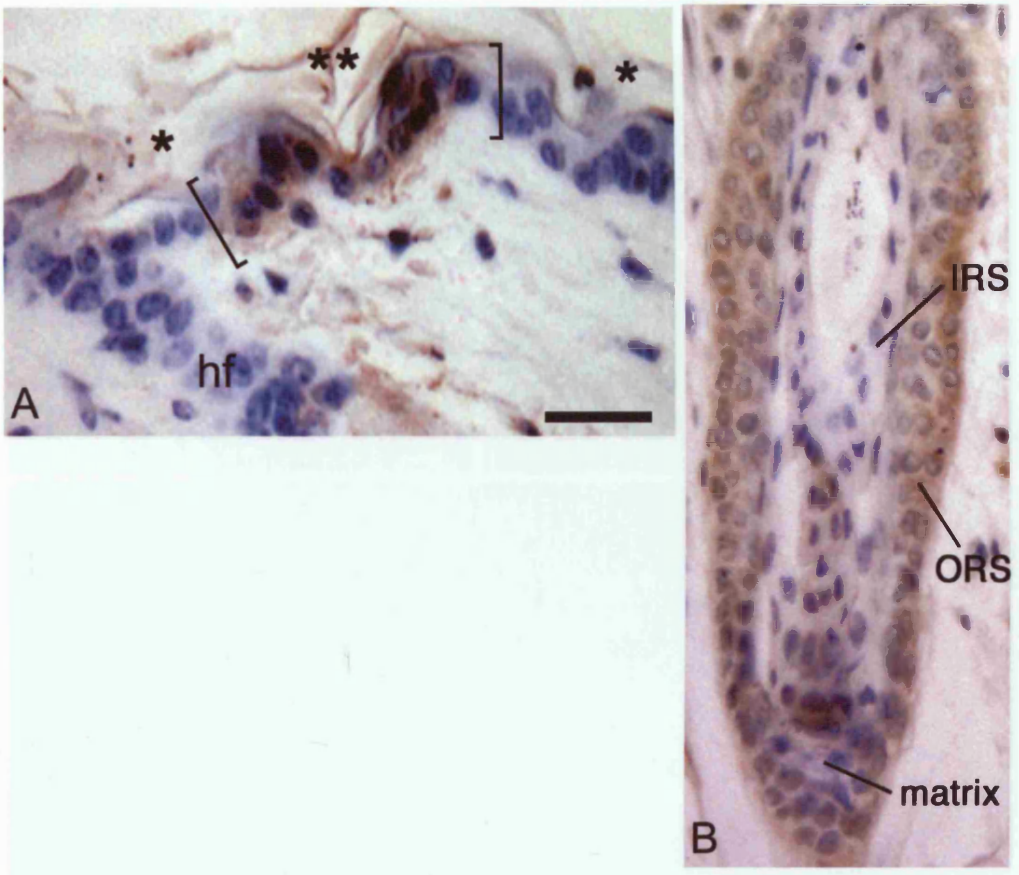

Figure 5.10) BM-derived cells form EPU-like structures in the regenerating epidermis, and BM-derived cellular columns were observed in hair follicles, *in vivo*.

Multiple adjacent GFP-positive cells (brown stain), resembling an EPU in the interfollicular epidermis, 4 days post wounding (A). The cornified layer of cells at the surface of the GFP-positive, EPU-like structure also expressed GFP (A; **), whereas cells from adjacent GFP-negative regions of epidermis did not (A; *). Columns of GFP-positive cells were observed in the outer root sheath (ORS), shown at 30 days post wounding (B). Note that some of the cells in the inner root sheath (IRS) and matrix are GFP-negative. Scale bar = 25μm.

Figure 5.11

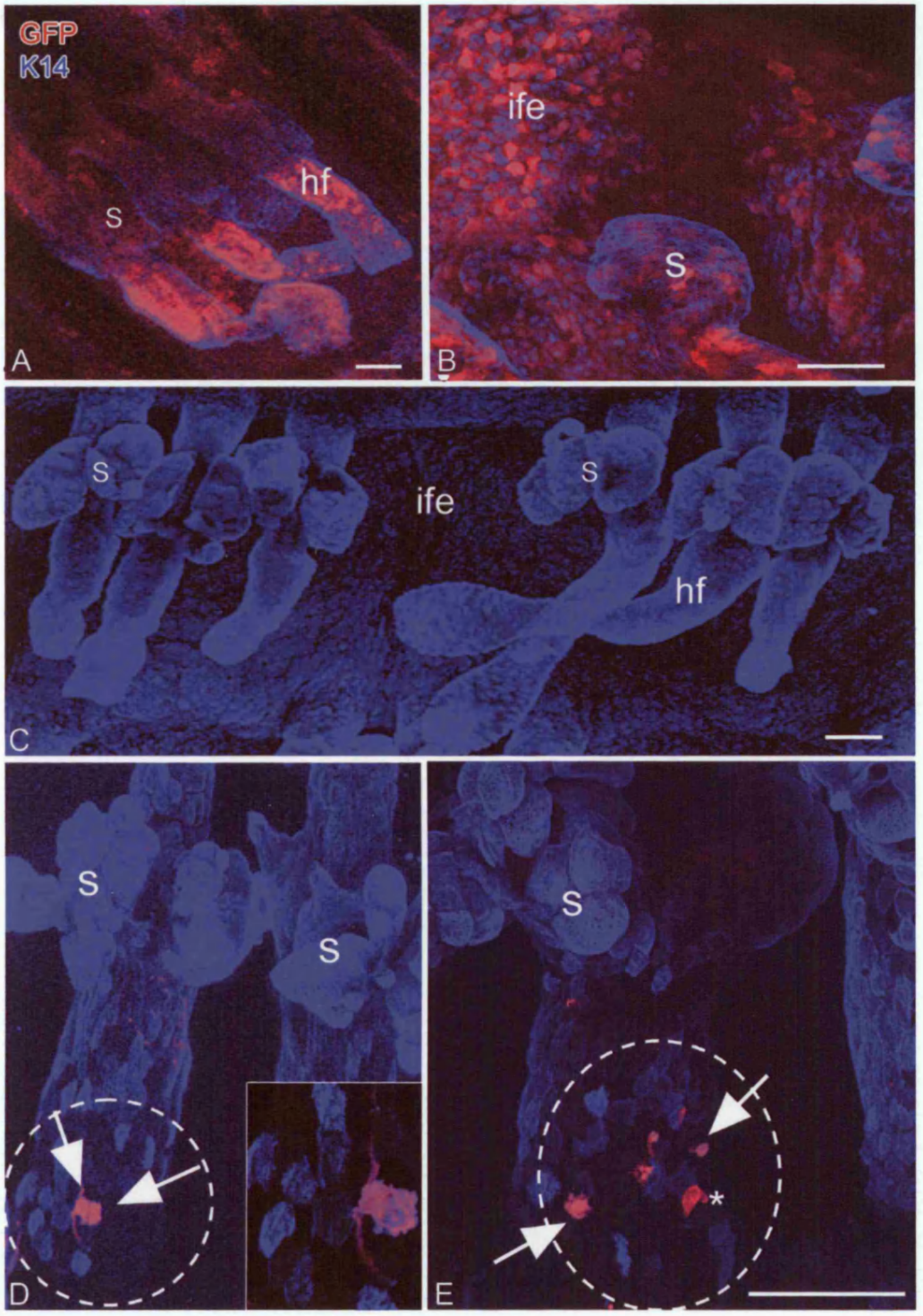

Figure 5.11) BM-derived keratinocytes were identified in epidermal wholemounts.

Co-localised GFP (Cy3-labelled, red) and K14 expression (Cy5-labelled, blue) resulted in a pink signal, and was detected in the interfollicular and follicular epidermis in tail epidermal wholemounts of control GFP transgenic mice (A, B). Control wild type epidermal wholemounts stained for both K14 and GFP displayed K14 expression but not GFP (C). In wild type mice transplanted with GFP-positive BM, keratinocytes of donor origin were identified by their co-expression of GFP and K14, and were frequently observed in the bulge region of hair follicles (D, and high power inset, E; arrows). K14-negative cells that expressed GFP were also observed and were believed to be inflammatory cells, shown here in the bulge region (E; *). s = sebaceous glands, ife = interfollicular epidermis, hf = hair follicle, the follicular bulge region is within dotted boundary. Scale bars = 50μm.

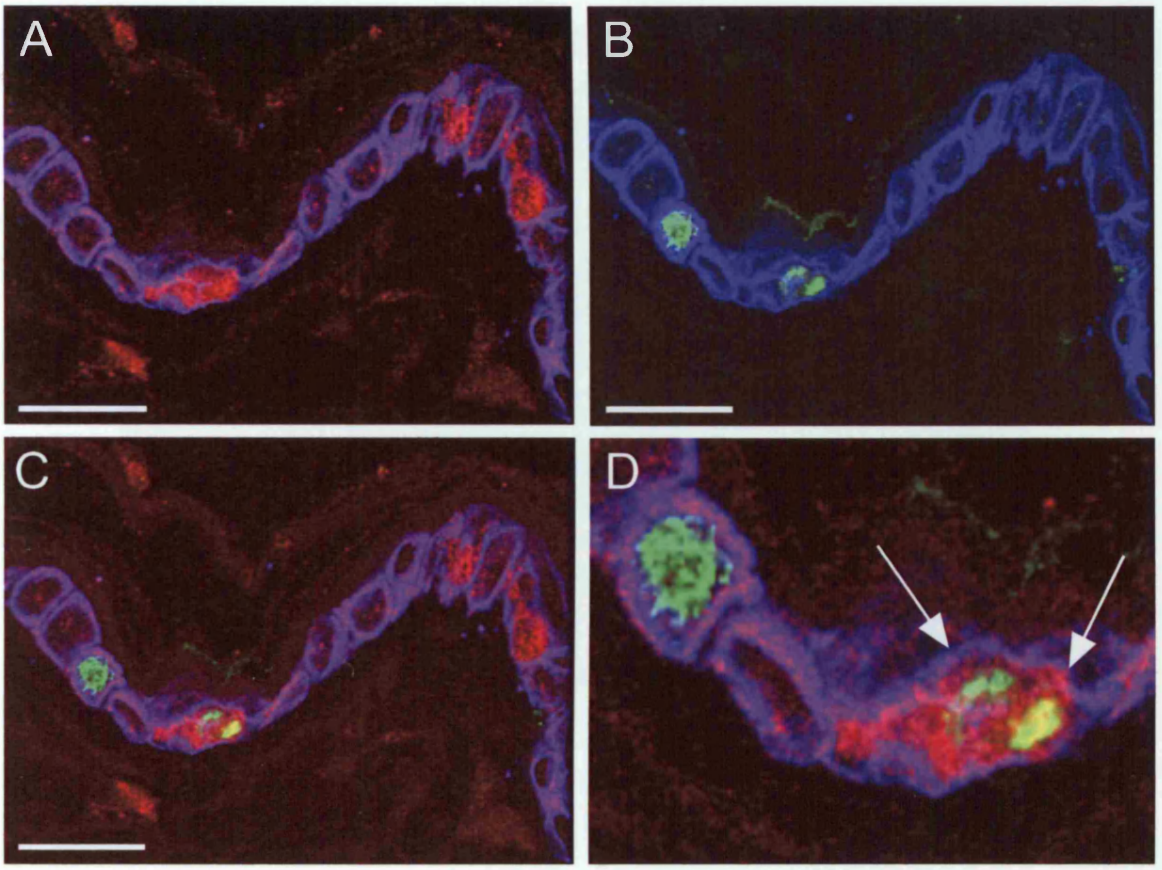

Figure 5.12) BM-derived keratinocytes can proliferate *in vivo*.
Mice transplanted with GFP-positive BM were injected with BrdUrd one hour prior to sacrifice, and skin was immunostained to identify K14 (Cy5-label, blue), GFP (Cy3-label, red), and BrdUrd incorporation (FITC label, green). GFP and K14 (A), BrdUrd and K14 (B), merge of A and B (C), and inset (D), illustrating a BM-derived, BrdUrd-positive keratinocyte in non-wounded epidermis 6 weeks post-transplant (arrows).

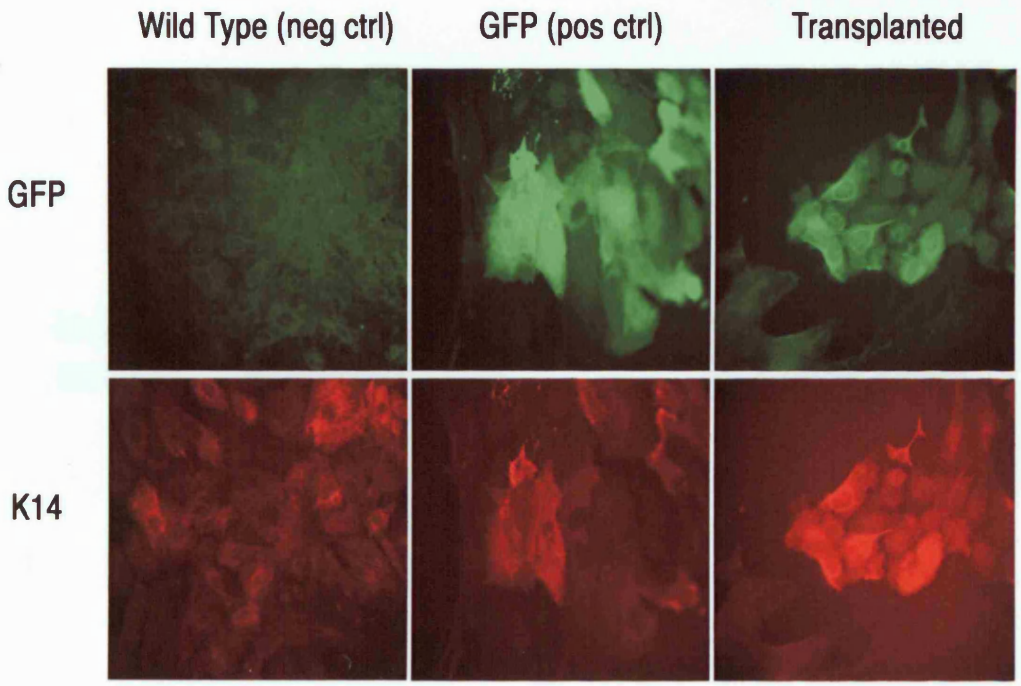

Figure 5.13) BM-derived keratinocytes can expand *in vitro*.
Keratinocytes were isolated from the epidermis, grown in culture for 3 weeks, and immunostained for GFP (FITC label, green) and K14 (Cy3 label, red). Those cells derived from wild type mice did not express GFP; those from GFP transgenic mice did express GFP, as expected. Some keratinocyte colonies from wild type animals transplanted with male GFP-positive BM cells, expressed GFP. All keratinocyte cultures express K14.

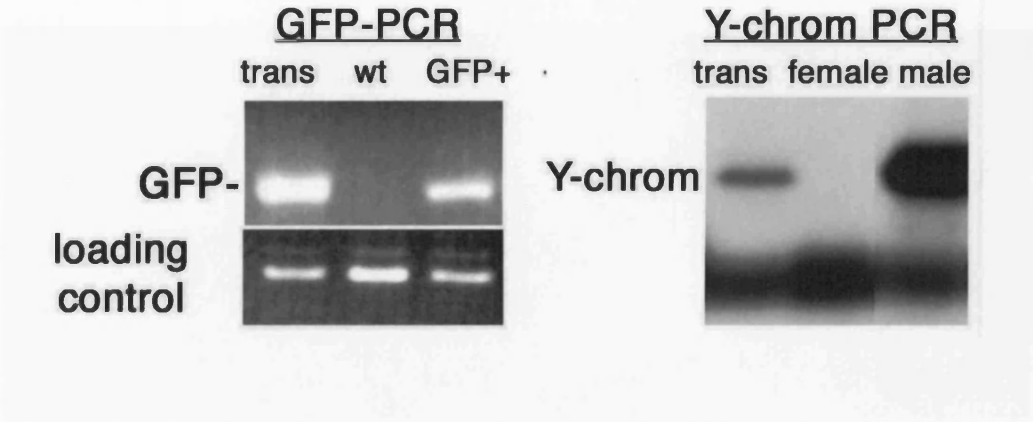

Figure 5.14) PCR analysis.

PCR analysis of GFP expression confirmed that GFP-immunoreactive keratinocyte colonies from wild type animals transplanted with male GFP-positive BM (trans) expressed GFP; wild type (wt) samples were negative for GFP; and GFP-positive (GFP+) cell samples were positive for GFP. In addition, PCR for Y chromosome expression showed that keratinocyte colonies from female wild type animals transplanted with male GFP-positive bone marrow (trans) were positive for the Y chromosome; keratinocytes from female wild type mice were negative; and keratinocyte colonies from male mice were strongly positive (PCR reactions were performed by Kairbaan Hodivala-Dilke).

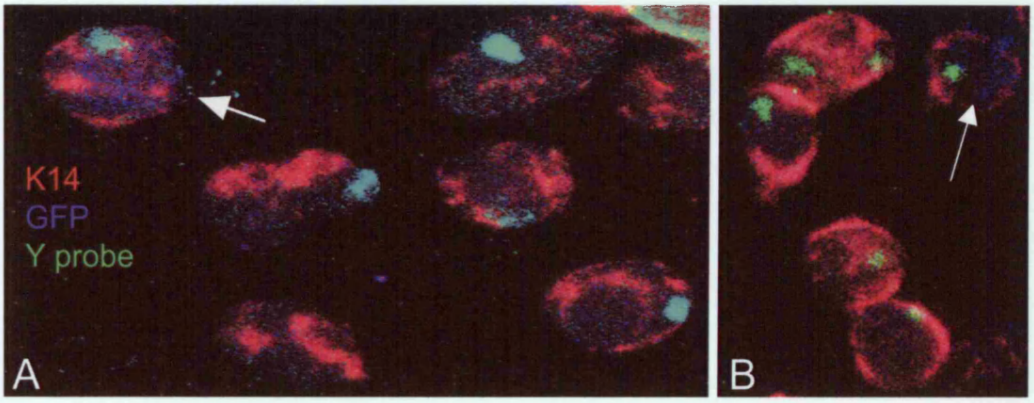

Figure 5.15) Transplanted BM cells engraft within the epidermis and form keratinocytes with no evidence of cell fusion.

Confocal analyses of epidermis (10 μm paraffin section) from male wild type mice transplanted with male GFP-positive BM cells, showed cells that express GFP (Cy5 label, blue) and K14 (Cy3 label, red), with a single Y chromosome (FITC label, green probe in nucleus), indicating that transplanted cells can form keratinocytes without evidence of fusion (A, B; arrows). No evidence of BM-derived keratinocytes with multiple Y chromosomes was observed.

CHAPTER VI

Discussion

6.1) General Summary

Adult stem cells were fundamentally believed to be restricted to the production of differentiated lineages within their native tissue. However, with the emergence of a wealth of evidence over recent years, it is becoming increasingly accepted that adult stem cells have the capacity to cross lineage boundaries and form adult lineages within multiple extraneous tissues. In this thesis I have shown that transplanted BM cells retain a large degree of plasticity and can give rise to multiple differentiated lineages in the mouse and human gastrointestinal tract and in the mouse epidermis.

In chapters III, IV and V, I have presented a number of novel observations and have established that:

(1) Transplanted BM cells can give rise to an extensive proportion of ISEMFs in the mesenchymal lamina propria of the mouse and human small intestine and colon following lethal irradiation.
(2) The number of BM-derived ISEMFs is significantly increased in a mouse model of colitis.
(3) Transplanted BM contains progenitors cells that can form endothelial cells and mural cells in the mouse colon.
(4) The number of BM-derived vascular-associated cells is significantly increased in a mouse model of colitis.
(5) Transplanted BM cells can form epidermal cell lineages in the hair follicles, IFE and sebaceous glands of the mouse skin.
(6) The number of BM-derived keratinocytes is significantly increased in the regenerating epidermis.

(7) BM-derived keratinocytes can proliferate *in vivo* and can form colonies *in vitro*.
(8) BM-derived keratinocytes can form without fusing to an indigenous keratinocyte.

Based upon these results, I have also postulated the following, although further studies will be essential to validate these points:

(1) BM-derived ISEMFs appear to be functional, as they exist in an interconnected cellular network in the lamina propria that spans from the base of the crypt to the intestinal lumen, thereby suggesting a proliferative capacity of these cells.
(2) BM-derived ISEMFs may contribute to the intestinal epithelial stem cell niche and assist in the maintenance of intestinal homeostasis by the paracrine secretion of specific growth factors and cytokines.
(3) BM cells may be therapeutic in the treatment of IBD via their formation of activated ISEMFs, which influence mucosal regeneration and remodelling.
(4) BM cells may be involved in neovasculogenesis in the inflamed mouse colon by differentiating to produce entire new blood vessels, and BM cells also appear to aid angiogenesis by contributing endothelial cells and mural cells to damaged blood vessels.
(5) My observation that transplanted BM cells frequently engraft within the postulated stem cell regions of the IFE and hair follicles, and can express a putative epidermal stem cell marker, suggests that BM cells contribute to the epidermal stem cell niche.
(6) BM cells may be therapeutic in re-epithelialisation of the wounded epidermis *via* their enhanced formation of keratinocytes in stressed tissue.

6.2) BM contribution to cells in the normal and inflamed gut

The intestinal stem cell is an empowering force, regulating homeostasis by altering the rate of cell proliferation and differentiation as regenerative demand dictates it. The intestinal stem cell can undergo self-renewal and programmed cell death and a constant stem cell number is therefore maintained in the base of the intestinal crypts to assure optimal gastrointestinal function. However, this cell does not act alone and the underlying mesenchymal cells and their secreted basement membrane factors in the subepithelial lamina propria are believed to directly regulate intestinal epithelial stem cell function by forming and maintaining the stem cell niche, and influencing the stem cells *via* the paracrine secretion of specific growth factors and cytokines. Little is understood about the origins of the intestinal epithelial stem cells and the underlying mesenchymal cells in the lamina propria, and therefore I have investigated the role of BM cells in their contribution to intestinal lineages, and the regenerative potential of BM-derived intestinal cells in a disease affecting gastrointestinal function.

6.2.1) Lack of BM-derived intestinal epithelial cells

BM cells have been reported to engraft within the mouse and human gastrointestinal tract and form cytokeratin-expressing epithelial cells in the gastric glands and the intestinal crypts and villi (Jiang et al., 2004; Korbling et al., 2002; Krause et al., 2001; Okamoto et al., 2002; Spyridonidis et al., 2004). I did not observe BM-derived epithelial cells in mouse or human gastrointestinal tissues, and so failed to reproduce these previous findings. In the mouse, this may be due to differences in the methods used i.e., transplantation of different sub-populations of fractionated BM, time elapsed between transplant and tissue analysis, the mouse strain used or experimental methods of donor cell detection.

Likewise, in the human, differences in the methods of tissue fixation, the degree of GvHD at the time of tissue biopsy, and differences in the time elapsed between transplant and biopsy may cause these variations in results.

6.2.2) BM-derived ISEMFs in the normal and inflamed colon

My observation that BM can form a large proportion of ISEMFs in the mouse and human small intestine and colon is a novel discovery (Brittan et al., 2002), although the ability of BM to differentiate to form myofibroblasts has subsequently been shown in several other tissues including the mouse lung, stomach, oesophagus, skin, kidney, adrenal glands and pancreas (Direkze et al., 2003; Direkze et al., 2004; Poulsom et al., 2001), and the human liver (Forbes et al., 2004). This data also supports recent studies showing that human MSCs can differentiate to myofibroblast-like cells *in vitro* (Ball et al., 2004; Emura et al., 2000; Kadner et al., 2002).

I also report, for the first time, that BM formation of ISEMFs within the mouse colon is enhanced in a model of colitis. This is supported by subsequent observations that BM upregulates its formation of myofibroblasts in models of disease and injury in other tissue systems (Direkze et al., 2003; Direkze et al., 2004; Forbes et al., 2004).

6.2.3) BM-derived vascular cells in the normal and inflamed colon

The contribution of BM cells to vascular lineages in several models of tissue injury and disease is well documented (section 1.12.5; Table 1.1), although I believe that this is the first report of a BM origin of vascular lineages in the mouse colon. Moreover, I report a statistically significant increase in the number of BM-derived vascular cells in a mouse model of experimental colitis, which further supports a therapeutic role of transplanted BM cells in inflammatory bowel disease, possibly by contributing to postnatal neovasculogenesis.

Emerging data shows that BM can provide long-term remission for patients with Crohn's disease (Kashyap and Forman, 1998; Lopez-Cubero et al., 1998; Soderholm et al., 2002; Talbot et al., 1998), which is supported by my own results. I also provide the first insight into the mechanisms by which BM cells may provide this remission from disease, namely in their formation of mesenchymal and vascular lineages to aid tissue regeneration.

6.3) BM-derived keratinocytes in the mouse epidermis

Several studies have reported a BM origin of keratinocytes in the epidermis of both mice and humans (Badiavas et al., 2003; Borue et al., 2004; Kataoka et al., 2003; Korbling et al., 2002; Krause et al., 2001), and an enhanced engraftment of BM cells within the regenerating mouse epidermis (Badiavas et al., 2003; Borue et al., 2004). My data support these observations of an increased number of BM-derived keratinocytes within the regenerating epidermis (chapter V; (Brittan et al., 2004)).

In addition, I report for the first time that BM-derived keratinocytes have the ability to proliferate both *in vivo* and *in vitro.* The therapeutic implications for this proliferative capacity of BM-derived cells are profound, as this confirms that transplanted BM cells can divide to produce daughter cells and thereby actively assist in tissue homeostasis and regeneration.

I have also shown that BM-derived keratinocytes can engraft within known epidermal stem cell niches in the IFE and hair follicles, which is evidence, albeit circumstantial, that BM-derived cells become located within the region of epidermis where they are encompassed by a regulatory, protective niche environment and are most likely to divide and differentiate to produce keratinocyte progeny. Furthermore, I have verified the expression of a putative epidermal stem cell marker, namely CD34, by BM-derived cells in the follicular bulge region.

The mechanism by which BM cells assume a differentiated non-haematopoietic cell phenotype is currently a matter of some debate, with claims that BM cells do not differentiate to produce non-haematopoietic lineages, but simply fuse with pre-existing adult cells within a tissue to form a heterokaryon (section 1.14). By analysing chromosomal content within BM-derived keratinocytes, I provide evidence that BM cells can form epidermal keratinocytes by *de novo* cell production, and do not

appear to fuse with an indigenous keratinocyte. Of all keratinocytes analysed (5 mice, 200 cells from each mouse), no cells were observed to contain an aberrant chromosome number indicative of cell fusion, and therefore I deem heterokaryon formation by transplanted BM cells in the mouse epidermis to be highly unlikely. However, cell fusion cannot be entirely discounted due to restrictions in the technique, although these restrictions may be overcome in the future by use of the Cre/lox recombinase system. Recent evidence showing BM cell fusion with cells in the liver, heart and brain is highly convincing (Alvarez-Dolado et al., 2003; Vassilopoulos et al., 2003; Wang et al., 2003; Weimann et al., 2003a; Weimann et al., 2003b), and I suggest that the mechanism of BM cell plasticity may be tissue-specific. A recent study has verified my own results that BM-derived keratinocytes can form in the mouse epidermis without fusion (Harris et al., 2004).

6.4) Future directions

The newly emerging science of adult stem cell plasticity and the potential for vast and diverse tissue regeneration by these cells is hugely exciting. The discovery that adult bone marrow stem cells can produce functional non-mural lineages with the potential to divide and form differentiated progeny highlights future aspirations for the use of adult stem cells in the treatment of pathological conditions. In this study I have shown that bone marrow stem cells may be therapeutic in the treatment of skin disease and in inflammatory bowel disease, which can lead to the development of colon cancer.

It is becoming clear that the degree of regenerative pressure within a tissue has a major influence on the level of BM cell engraftment and contribution to adult cells of that tissue (section 1.13). I have confirmed that BM engraftment into the mouse colon and epidermis is significantly enhanced in disease or injury using models of experimental colitis and cutaneous wounding, respectively, and it is therefore possible that BM cells are involved in the regeneration of these damaged tissues.

In the lamina propria of both the non-inflamed and inflamed colon, BM-derived ISEMFs were frequently present in cellular columns spanning from the base of the crypt to the intestinal lumen, which is highly suggestive that these cells divide to produce differentiated progeny. Unfortunately, due to restrictions in the Home Office Project licence used in this study, it was not possible to inject the TNBS-treated mice with BrdUrd to label the dividing cells and therefore investigate the proliferative capacity of BM-derived cells in colitis. However, a new project licence has recently been written outlining the above experiment, which will be investigated within the Histopathology Unit at CRUK in the near future.

Future studies should involve elucidation of the stimulatory factors and molecular pathways that influence a BM cell to travel to and engraft within a tissue, and differentiate to form a defined lineage. It is likely that BM cells respond to aberrant levels of secretion of specific growth factors or chemokines by a tissue in disease or injury. For example, the intestinal myofibroblasts are a source of many pro-inflammatory cytokines and growth and differentiation factors, and are considered to be major role players in the initiation and regulation of intestinal immunity in normal and inflammatory conditions, including Crohn's disease (Fiocchi, 1997; Otte et al., 2003; Pang et al., 1994; Roberts et al., 1997; Strong et al., 1998). Therefore, the intestinal myofibroblasts appear to be likely candidates for the cells that stimulate the cells of the BM to home-to and engraft within the inflamed gut, and aid tissue regeneration by initiating a T-cell response, and as my data suggests, by differentiating to form both mesenchymal and vascular lineages.

It is important to define the specific subpopulations of cells present in the BM that can form each specific lineage in adult tissues. At the moment it is not clear if the BM contains a single primitive stem cell population that can produce multiple adult lineages within numerous tissues, or whether multiple stem cell populations are present within the BM each pre-programmed to differentiate and form cells within a specific tissue. It is possible that BM cells respond to external cues from a tissue in stressed conditions, and then migrate to and engraft within this tissue whereupon they initiate differentiation to a required cell type. Future studies involving purification and transplantation of distinct BM stem cell populations will assist the identification of innate differentiation pathways, and will shed light upon the above problem. Indeed, in the Histopathology Unit at CRUK, studies wherein mouse BM is sorted into defined haematopoietic- and mesenchymal-expressing populations prior to transplantation into an irradiated host are currently underway.

Knowledge of the distinct progenitor cells within the BM will also permit the manipulation of these cells by introduction of therapeutic genes that

can then be targeted directly to a specific diseased tissue. For example, the pro-inflammatory cytokines interleukin-1β and tumour necrosis factor-α (TNFα) are upregulated in Crohn's disease (Mahida et al., 1989; Reinecker et al., 1993), and these have been shown to drive the proliferation of human colonic myofibroblasts *in vitro* (Jobson et al., 1998), which can lead to fibrosis and scarring (Aigner et al., 1997; Graham, 1995; Isaji et al., 1994; Kinzler and Vogelstein, 1998; Martin et al., 1996). It would therefore be interesting to transplant BM cells that have been genetically-modified to express anti-inflammatory cytokines such as interleukin-10 or anti-TNFα antibodies, and investigate whether these cells can differentiate to form ISEMFs with anti-inflammatory properties in our model of TNBS colitis, and thereby alleviate the disease.

So, to summarise, I believe that the results presented in this thesis are a further step forward in the understanding of the functional relevance of adult stem cell plasticity. The BM proves an excellent source of readily accessible adult stem cells, and I have now ascertained that adult BM contains progenitor cells for multiple non-haematopoietic lineages, namely, the intestinal myofibroblasts, the endothelial cells and mural cells within blood vessels, and the keratinocytes and sebocytes of the epidermis. Transplanted BM may prove therapeutic in the treatment of pathological conditions of the skin and inflammatory disease of the intestine.

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