STUDIES INTO CHARACTERISTICS OF CANCER STEM CELLS AND THEIR ROLE IN THE ORIGIN OF EPITHELIAL TUMOURS

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A thesis submitted for the degree of

Doctor of Philosophy

at the University of London

February 2008

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ABSTRACT

Tumours are organised in a cellular hierarchy, originating in and maintained by a minority of cells with stem cell characteristics, so-called 'cancer stem cells'. The Side Population (SP) phenotype, distinguished by its ability to efflux the nucleic acid dye Hoechst 33342, as well as surface markers CD44 and CD133 were evaluated as potential cancer stem cell markers in human colorectal cancer cell lines and primary adenocarcinomas by testing sorted cell fractions for phenotypical and behavioural stem cell-typical characteristics such as clonogenicity or sphere formation *in vitro*, tumorigenicity *in vivo*, and multilineage differentiation potential *in vitro* and *in vivo*. My results indicate that in human gastrointestinal cancer cell lines, the SP, albeit containing cells with stem cell characteristics, is not enriched in stem cells as compared to the non-SP and thus does not present a useful tool for the isolation and identification of stem cells. Similarly, no stem cell characteristics were detected in the CD44+, CD133+, or double positive cell fractions of primary human colorectal carcinomas.

Next, tumour-initiating capacities of transplanted cells and the reciprocal effects between transplanted cells, engrafted in the skin, and the local microenvironment were investigated in human kidney transplant patients and a BM-transplanted mouse model. We were able to show that donor-derived cells engraft as epithelial cells in normal and tumour epithelium, and contribute to stromal and endothelial tumour components without any events of cell fusion in both, human and mouse skin tumours. Observations of cell clusters and proliferation activity suggested that cells from BMTs may be able to engraft at earlier differentiation stages than cells from solid organ transplants. The sporadic distribution of engrafted cells and the absence of donor-derived tumours in our large scale human study indicated that cells from kidney transplants do not engraft as stem cells or cancer stem cells in the skin, even upon HPV-infection. Rather than playing an active role in carcinogenesis or tissue remodelling, donor-derived cells appear to imitate their surrounding cells in morphology and function. Following the patterns of engraftment and tumour development strongly suggests that the local cellular host microenvironment exerts more influence on the outcome phenotype than the effect of genetic pre-disposition of engrafted transplanted cells.

Finally, on a histochemical and molecular level I show that squamous cell carcinomas (SCCs) and basal cell carcinomas (BCCs) show distinct developmental dynamics, presumably reflecting their origin in different stem cell pools. Moreover, evidence is provided for a common developmental origin of outer root sheath and BCC, presumably in the stem cells of the hair follicle bulge. Histochemical analysis demonstrates that the companion layer is genetically continuous with the inner root sheath lineages and can be viewed as its outermost layer. We also suggest that BCCs and their stroma may share a common origin.

Together, these results provide insight into the characterisation of cancer stem cells and the role of cancer-originating cells in tumour development.

ACKNOWLEDGEMENTS

First and foremost, my warmest and sincerest thanks to Nick! Thank you for giving me the chance, for your guidance, support and motivation. You are my hero! I am also greatly indebted to Malcolm and Bill – thanks for your patience, efforts, encouragement, and kicks when I needed them!

Thanks to everyone in the Histopathology lab. I can't begin to explain how grateful I am for having had the chance to meet and work with you all. There is probably not another lab out there with an atmosphere this supportive, caring, and not to forget – social. Simon, Toby, after having had to work alongside you, how can any other place not feel incredibly boring? Thank you Stuart for your friendship and support! Can't wait for all the future rounds of golf. George, thank you for everything, you are possibly the most helpful man in the world. Thank you Rosemary and Richard for your advice and approachability.

Thank you to my familiy and friends! My dear grandparents, I promise, one day your patience will pay off and I will get a proper job. Mama, thank you for sending me on the path, making it all possible and being there always!! Papa, my memories of you and your spirit have and will always guide me – this thesis is dedicated to you!

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AML	acute myeloid leukemia
BCC	basal cell carcinoma
AK	actinic keratosis
BK	bowenoid keratosis
BADJ	bronchioalveolar duct junction
BM	bone marrow
BrDU	bromodeoxyuridine
CC	cholangiocarcinoma
CFU	colony forming unit
CIS	carcinoma in situ
CML	chronic myeloid leukemia
CSC	cancer stem cell
DMBA	7,12-dimethylbenz(a)anthracene
EPU	epidermal proliferative unit
FACS	fluorescence-activated cell sorting
5-FU	5-fluorouracil
GvHD	graft versus host disease
HCC	hepatocellular carcinoma
IFE	interfollicular epidermis
IRS	inner root sheath
LRC	label retaining cell
ORS	outer root sheath
SC	stem cell
SCC	squamous cell carcinoma
SP	side population
TAC	transit amplifying cell

CHAPTER I

GENERAL INTRODUCTION

1.1 ADULT STEM CELLS

Stem cells are primitive cells that are capable of self-renewal, proliferation and differentiation into mature cells. First identified in the inner mass of the blastocyst in embryos, stem cells have also been and continue to be found in adult tissues. Whereas embryonal stem cells display pluripotentiality and can constitute any organ in the body, adult stem cells are regarded as multipotential and thus limited to differentiate into cell types of the tissue in which they reside. However, there is accumulating evidence that adult stem cells may show plasticity and are able to engraft fully functionally into tissues outside their lineage. The ability of a single cell to give rise to a large family of descendants, containing all the lineages normally found in that cell's tissue of origin is considered a reasonably robust proof of 'stemness'. If cell migration occurs in a tissue, stem cells are at the beginning of the flux. Particularly where cell flux is unidirectional, such as in the epidermis, stem cells are at the beginning of the cell flux in the basal layer, with cells being shed at the surface. Adult stem cells with capacities for self-renewal and multilineage potential were first recognised in the bone marrow (BM) by Till and McCulloch who described cells that gave rise to multilineage haematopoietic colonies in the spleen (colony forming units – spleen [CFU-S]) (Till *et al.* 1961).

Stem cells, though highly clonogenic, are considered by most to be proliferatively quiescent. They cycle infrequently, giving rise to highly proliferative transit-amplifying cells (TAC) which are capable of limited self-renewal. After at the most, three to four cell divisions, their progeny differentiate into one of the mature cell types. Due to these proliferative habits, stem cells in animals that incorporate DNA synthesis labels such as

tritiated thymidine or bromodeoxyuridine (BrdU) tend to remain 'labelled' for longer than TACs whose more rapid cycling soon dilutes the label below detection levels. Thus, the identification of label-retaining cells (LRCs) is often used as a stem cell marker (Braun *et al.* 2004).

1.1.1 The stem cell niche

Stem cells are located in restrictive environments called 'niches' that play an integral part in stem cell survival and function. According to the "stem cell niche" hypothesis, interactions with the niche cells are crucial to the self-renewal process of stem cells (Schofield 1978), suggesting that stem cells may be regulated by their environment, rather than by intrinsic programming.

Characterisation of the components comprising the stem cell niche is difficult, partly due to the indistinct phenotype of stem cells, which prevents them from being identifiable in many adult tissues. Essentially, the stem cell niche consists of a sheath of mesenchymal cells and extra-cellular substances which surrounds the epithelial stem cells and provides an optimal microenvironment by paracrine secretion of growth factors and cytokines. The extracellular matrix is thought to play a special role in maintaining homeostasis by regulating adhesion to stem cells, thereby regulating retention and loss of stem cells from the niche. Due to the hierarchical and migratory nature of cells in the differentiation process, the niche is most likely situated at the origin of the cellular flux. When stem cell progeny leave the niche they differentiate to become transit-amplifying cells (Figure 1.1, pg.21).

Given the immense influence the niche exerts on the development and behaviour of stem cells, it is clearly equally, if not more important, to understand and unravel the cells involved and the regulatory processes in the niche than it is to understand the nature of stem cells themselves. Indeed, controlling factors for setting up the niche microenvironment are rapidly becoming elucidated: in the gut for example, Wnt signalling is

crucial to stem cell homeostasis (Sancho *et al.* 2004), and Wnt inhibitors such as Dikkopf-1 could become important anti-cancer agents (Kuhnert *et al.* 2004).

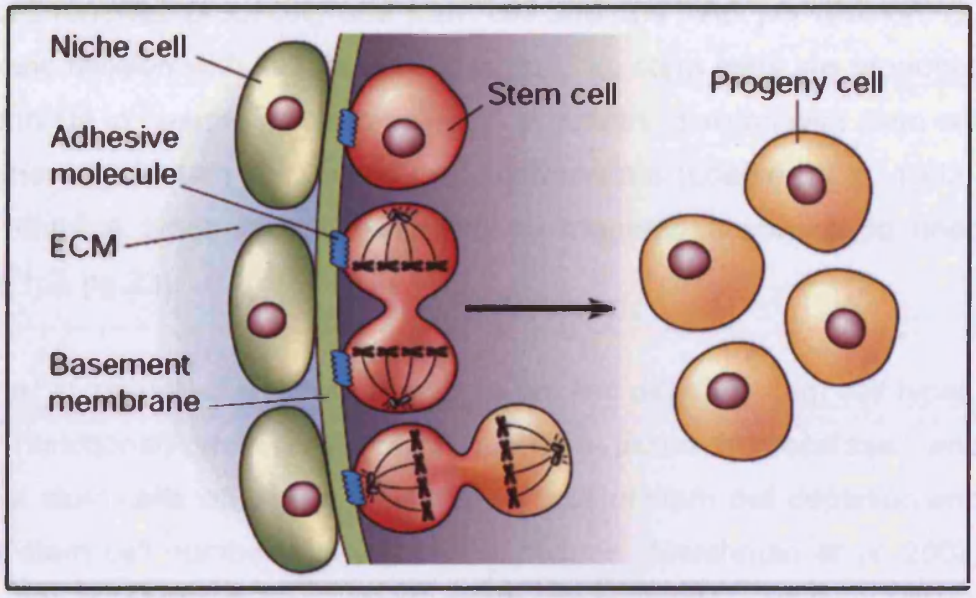

Figure 1.1. The stem cell niche. Stem cell behaviour is governed by niche cells which provide an optimal microenvironment by the secretion of extracellular matrix, signalling molecules, and cell-to-cell contact. Stem cell progeny can leave the niche as transit amplifying cells (yellow) and undergo the pathway to terminal differentiation (bulk of yellow cells). Diagram taken from (Spradling *et al.* 2001).

1.1.2 Stem cell division

Three different mechanisms for division of adult stem cells have been proposed: (1) asymmetric division is thought to be the major mechanism for stem cell renewal, producing one stem cell and one TAC per division; (2) symmetric division with self replication where two stem cells are produced that continue to operate as stem cells; (3) symmetric division with stem cell loss, where both stem cells move on to differentiate (Loeffler *et al.* 1993). These division types may coexist and be triggered according to need (Figure 1.2, pg.23).

Potten *et al.* proposed a theory that there are two different stem cell types: actual (functional) stem cells which maintain tissue homeostasis, and potential stem cells which are mobilised in case of stem cell depletion and restore stem cell number by symmetrical division (Marshman *et al.* 2002, Potten *et al.* 1990, Potten *et al.* 1997). To achieve self-renewal, on average, each stem cell division gives rise to one replacement stem cell and one TAC by asymmetric cell division. Thus, the probability (P) of self-renewal is 0.5, and any upward shift in p will lead to a rapid escalation in stem cell numbers – a likely event in many cancers (Clevers 2005). Indeed, disruption of asymmetric neuroblast cell division in *Drosophila melanogaster* leads to lethal, expansive, tumour-like lesions when such cells are transplanted to adult hosts (Caussinus *et al.* 2005).

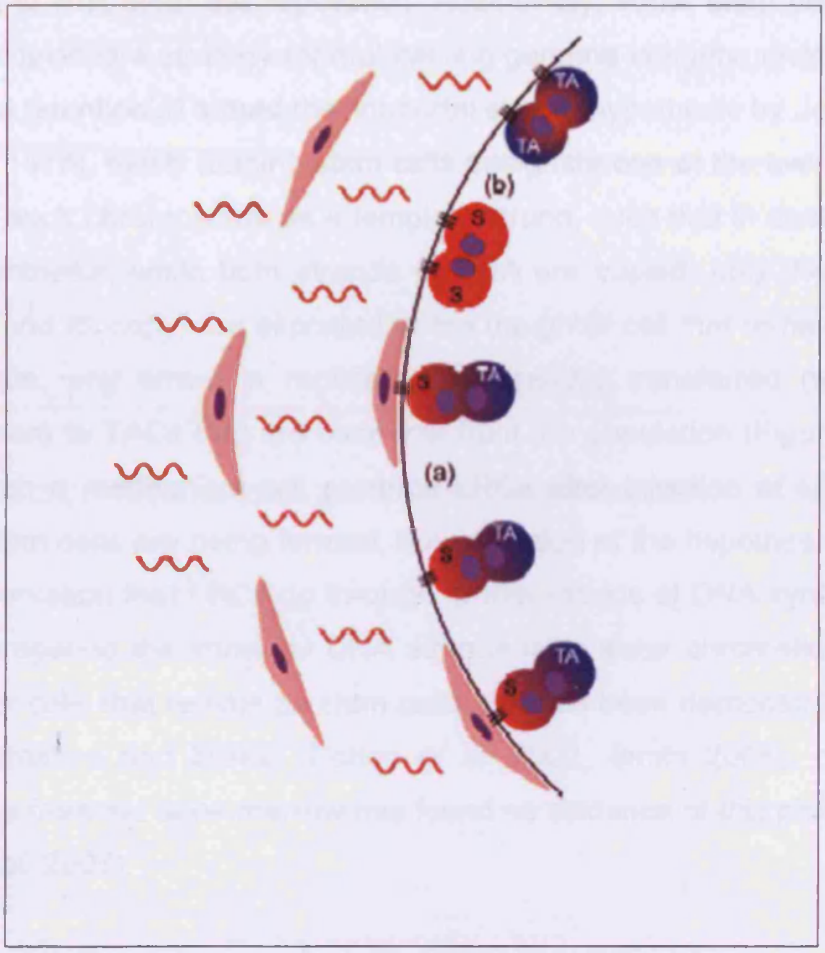

Figure 1.2. Possible outcomes of stem cell division. (a) in asymmetric division, one stem cell progeny leaves the niche to undergo terminal differentiation and one cell remains in the niche. (b) When stem cells undergo symmetrical division, they either produce two identical stem cell progeny or two transit amplifying cells. (Diagram courtesy of Matthew Lovell).

DNA synthesis is error prone and thus, teleologically, it would seem prudent to limit stem cell replication. Additionally, some stem cells appear to have devised a strategy for maintaining genome integrity; another cause for 'label retention'. Termed the 'immortal strand' hypothesis by John Cairns (Cairns 1975), newly forming stem cells designate one of the two strands of DNA in each chromosome as a template strand, such that in each round of DNA synthesis, while both strands of DNA are copied, only the template strand (and its copy) are allocated to the daughter cell that remains a stem cell. Thus, any errors in replication are readily transferred (within one generation) to TACs that are soon lost from the population (Figure 1.3, pg. 25). Such a mechanism will produce LRCs after injection of DNA labels when stem cells are being formed, but validation of the hypothesis requires the observation that LRCs go through further rounds of DNA synthesis, yet still segregating the immortal DNA strands from sister chromatids into the daughter cells that remain as stem cells; this has been demonstrated in the small intestine and breast (Potten *et al.* 2002, Smith 2005), but recent evidence from the bone marrow has found no evidence of this phenomenon (Kiel *et al.* 2007).

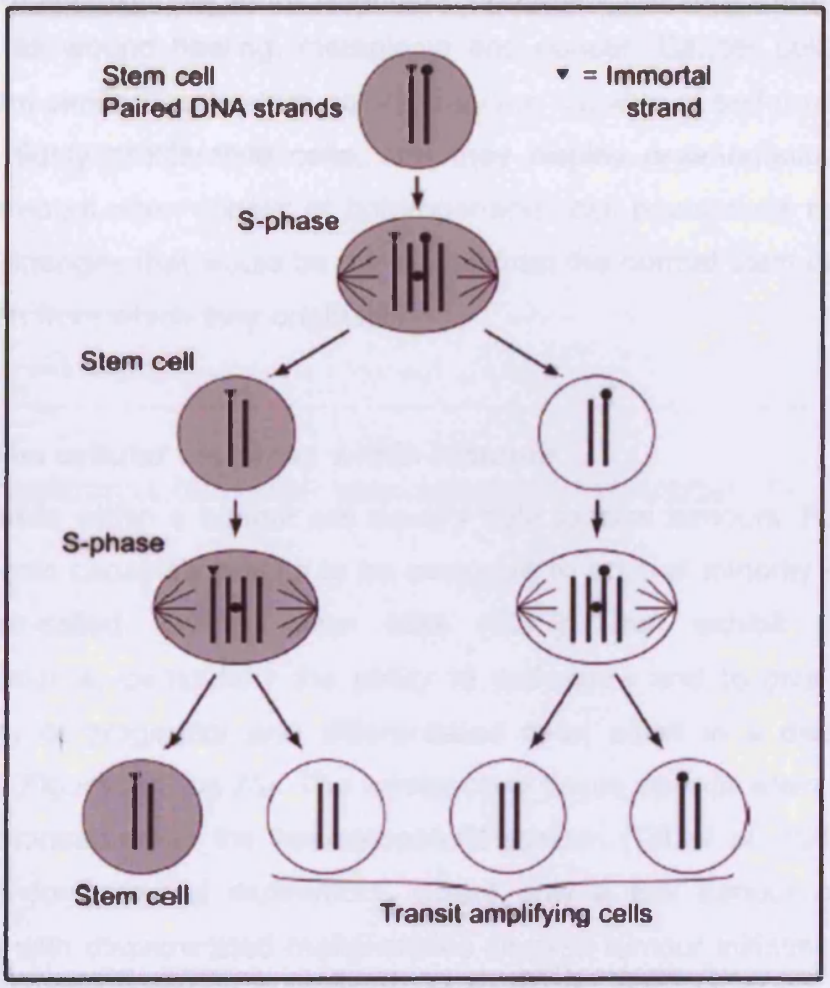

Figure 1.3. The immortal strand hypothesis, proposing how the genomic integrity of stem cells can be preserved. Newly synthesized strands of DNA are always allocated to TACs, and stem cells retain the immortal (template) strand. The template strand of each chromosome is established when stem cells are created. (Diagram taken from Burkert *et al.* 2006).

1.2 CANCER STEM CELLS

For many years it has been clear that stem cells feature in processes as diverse as wound healing, metaplasia and cancer. Cancer cells harbour significant similarities to stem cells – they are capable of self-renewal, give rise to highly proliferative cells, and they display organogenic capacity, since tumours often consist of heterogeneous cell populations resembling the cell lineages that would be generated from the normal stem cells within the organ from which they originate.

1.2.1 The cellular hierarchy within tumours

Not all cells within a tumour are equally able to form tumours. Rather, the tumorigenic capability seems to be exclusive to a small minority of tumour cells, so-called 'cancer stem cells (CSC)', that exhibit stem cell characteristics, particularly the ability to self-renew and to give rise to a hierarchy of progenitor and differentiated cells, albeit in a disorganised manner (Figure 1.4, pg.28). The existence of tissue specific stem cells was first demonstrated in the haematopoietic system (Till *et al.* 1961). In an ethically controversial experiment, where only a few tumour cells from patients with disseminated malignancies showed tumour initiating capacity when injected back into the same patients, Brunschwig *et al.* demonstrated for the first time that tumour cells seem to be hierarchically-organised (Brunschwig *et al.* 1965). Other evidence for a hierarchical organization within a tumour came from the observation that only 1-4% of lymphoma cells were able to form colonies *in vitro* or generate spleen colonies when transplanted *in vivo* (Bruce *et al.* 1963). Likewise, studies of human tumours revealed clonogenic potentials in the range of 0.0001-1% *in vitro* (Hamburger *et al.* 1977, Park *et al.* 1971). These observations led to the hypothesis that leukaemia and other malignancies must be maintained by a small population of CSCs with the potential for self-renewal and extensive proliferation (Griffin *et al.* 1986, Hamburger *et al.* 1977, McCulloch 1983).

With the development of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice for the xenografting of human tumours, came the first good *in vivo* evidence for the existence of CSCs (Bonnet *et al.* 1997). Bonnet and Dick showed that only a small subset of AML cells was capable of producing leukaemic progenitors and leukaemic blasts upon transplantation into immunodeficient mice, resulting in a disease phenotype identical to the donor (Bonnet 2005). Since the first account of CSCs in leukaemia, they have also been demonstrated in various solid tumours. In the breast, the existence of stem cells was inferred by the clonogenicity (ability to form mammospheres) and by the multipotentiality of a subpopulation of murine mammary cells (Al-Hajj *et al.* 2003, Dontu *et al.* 2003). The existence of CSCs has been confirmed in tumours of the brain, breast (Al-Hajj *et al.* 2003), colon (O'Brien *et al.* 2007, Ricci-Vitiani *et al.* 2007), liver, skin (reviewed in (Perez-Losada *et al.* 2003)) and others.

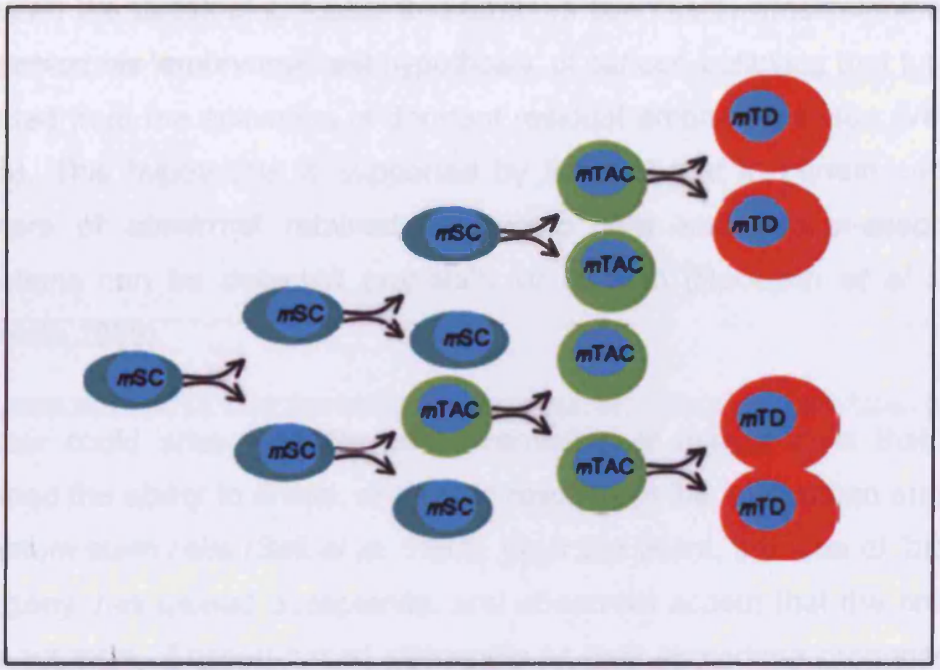

Figure 1.4. The generation of cellular heterogeneity in a tumour. Dysregulation of asymmetrical cell division in a malignant stem cell (mSC) leads to increasing numbers of mSC, and haphazard combinations of symmetric and asymmetric divisions will produce malignant transit amplifying cells with limited proliferative potential and malignant terminally-differentiated cells with no proliferative potential. (Figure taken from Burkert *et al.* 2006).

1.2.2 The cellular origin of cancer stem cells

A connection between tissue development and tumour formation was first proposed in 1855 by Rudolph Virchow. Based on histological similarities between the developing foetus and tumours such as teratocarcinomas, he suggested his 'embryonal-rest hypothesis' of cancer, believing that tumours resulted from the activation of dormant residual embryonic tissue (Virchow 1855). This hypothesis is supported by the fact that in certain cancers, clusters of abnormal retained embryonic cells and cancer-associated mutations can be detected prenatally or at birth (Beckwith *et al.* 1990, Wiemels 1999).

Cancer could arise from the dedifferentiation of mature cells that have retained the ability to divide, or it could result from the 'maturation arrest' of immature stem cells (Sell *et al.* 1994). Over the years, the idea of 'blocked ontogeny' has gained acceptance, and observers accept that the arrested differentiation of tissue-based stem cells or their immediate progenitors is closely linked to the development of not only teratocarcinomas and haematological malignancies, but also carcinomas. Some of the most frequent tumours occur in tissues with a high cell turnover such as the skin and the epithelial lining of the gastrointestinal tract. It is argued that in these tissues the stem cells are the only cells with sufficient lifespan to acquire the requisite number of genetic abnormalities for malignant transformation. Additionally, with a self-renewal mechanism already in place, seemingly fewer alterations are required to change normal stem cells into CSCs. In the now classical two-stage model of mouse skin carcinogenesis, severely delaying the interval between 7,12-dimethylbenz(a)anthracene (DMBA) initiation and the application of the phorbol ester promoter had no bearing on subsequent tumour yield – strongly suggestive of an origin in a long-lived cell, an epidermal stem cell (Berenblum *et al.* 1949). This has again been experimentally demonstrated in mouse skin with an experiment where the interval between chemical cancer initiation and promotion proved that the only cells with a sufficient lifespan to have been present at the time of initiation are SCs (Van Duuren *et al.* 1975).

A stem cell origin of cancer is often inferred from the location of the tumour or its dysplastic antecedents, which does not preclude an origin from a more committed progenitor cell in the same location. For example, gliomas in the human brain are frequently found in a periventricular location, and experimental brain tumours induced by ethyl nitrosourea (ENU) are usually found in the subventricular zone – both major CNS stem cell areas (reviewed in (Sanai *et al.* 2005)). In the mouse lung, the bronchioalveolar duct junction (BADJ) is a major stem cell zone for the airway, and *ras*-induced adenocarcinomas arise in this area containing clusters of cells with the same stem cell characteristics (Kim *et al.* 2005). In the liver, the consensus view is that a variety of intrahepatic stem cells can give rise to hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) (Alison *et al.* 2005). Many liver tumours arise in a cirrhotic setting at a time when hepatocyte senescence triggers a reserve stem cell compartment to give rise to bipotential hepatic progenitor cells (HPCs); the fact that many liver tumours have features of both HCC and CC, combined with the presence of numerous HPCs, is consistent with an origin from HPCs or their antecedents. Indeed, gene expression profiling has identified a subset of HCCs with a very poor prognosis that had a profile consistent with an origin from HPCs (Lee *et al.* 2006). In the cornea, tumours appear to arise in the peripheral zone, which is the limbal stem cell zone, again suggestive of a stem cell origin (Waring *et al.* 1984). In the colon too, inspection of the very earliest dysplastic lesions suggests an origin from a mutated basally-situated stem cell that 'hitchhikes' its way to clonal dominance in a crypt of multiple stem cells through a process known as 'niche succession' to form a moncryptal adenoma (Calabrese *et al.* 2004, reviewed in (Leedham *et al.* 2005)). Though it is a widely held view that tumours are clonal populations, studies of early familial adenomas both in patients with familial adenomatous polyposis (FAP) and those in the mouse model ($Apc^{Min/+}$) suggest that the majority of nascent tumours are in fact polyclonal, requiring short-range interactions between adjacent initiated crypts: 'collision' tumours as a cause of this apparent polyclonality were ruled out

by genetically manipulating the $Apc^{Min/+}$ mouse to have far fewer tumours than normal (Thliveris *et al.* 2005).

In many haematological malignancies an origin from normal HSCs is indicated by the fact that one of the oncogenic alterations (presumably the earliest), can also be found in a variety of haematopoietic progenitor and differentiated cell types in patients in remission – again suggestive of an origin in a multipotential stem cell that subsequently accrues further mutations that lead to malignancy. This can be observed in acute myeloid leukaemia (AML) where the balanced translocation t(8;21) leads to the fusion transcript *AML-ETO* being found in many non-leukaemic cells (Miyamoto *et al.* 2000). Likewise, in chronic myeloid leukaemia (CML) where 95% of affected individuals have the Philadelphia chromosome, the *BCR-ABL* fusion transcript can also be found in otherwise normal mature blood cells (Cobaleda *et al.* 2000).

SCs seem to be not only involved in but even necessary for cancer development. In the skin for example, the stem cells of the hair bulge have been shown as a target for UV-induced mutagenesis and the source of SCCs as mice that had their interfollicular epidermis (IFE) removed, still developed SCC in response to UVB radiation (Faurschou *et al.* 2007). CD34 is expressed in the hair bulge of mice and considered as a potential epidermal stem cell marker. Knockout mice lacking the CD34-gene locus also fail to develop papillomas in initiation-promotion experiments (Trempus *et al.* 2007).

While it seems likely that most CSCs originate from transformed normal stem cells, they could also have their origins in more committed cells caused through, for example, reactivation of self-renewal mechanisms. Induction of stem cell-like features in more mature cells may permit a CSC to emerge from a cell not originally a tissue stem cell (Passegue *et al.* 2003). For example, when HSCs and myeloid progenitor cells were transduced with a leukemogenic *MLL-ENL* fusion gene and the cells transplanted *in vivo*, AML arose from both populations with the same

latency (Cozzio *et al.* 2003). Moreover, their immunophenotype and gene expression profiles suggested maturation arrest at identical stages of the differentiation pathway. Likewise, the *MOZ-TIF2* oncogene can confer leukaemic stem cell properties (which could be continuously propagated in liquid culture and result in AML after transplantation) upon granulocyte-monocyte progenitors (Huntly *et al.* 2004). Interestingly, the *BCR-ABL* oncogene was not able to do this, indicating a hierarchy of leukaemia-associated protooncogenes. The stem cell profile may also change during the course of a disease: in patients with CML in blast crisis, granulocyte-monocyte progenitors can become self-renewing CSCs, a change associated with Wnt pathway activation (Jamieson *et al.* 2004).

1.3 CONTROL OF STEM CELL FUNCTION

1.3.1 Wnt/β-catenin pathway

The Wnt/β-catenin pathway is involved in all of the stem cell-defining characteristics: self-renewal, proliferation and differentiation, and has been identified most frequently among cancers of the colon (reviewed (Reya *et al.* 2005)). The signalling cascade is initiated when extracellular Wnt molecules bind to frizzled, their receptor on the cell membrane. This causes an inhibition of the β-catenin destruction complex and consequently an accumulation of β-catenin, which then translocates to the nucleus where it activates the transcription of proliferation-promoting genes *via* binding to LEF/TCF transcription factors (Figure 1.5, pg.34). *In vitro* cultures of HSCs overexpressing stabilized β-catenin proliferate with a 100-fold increase (Reya *et al.* 2003) and addition of purified Wnt3a protein renders *in vitro* cultures of HSCs 6-fold more likely to proliferate and reconstitute the haematopoietic system of irradiated mice, but less likely to differentiate and express differentiation markers (Willert *et al.* 2003).

Wnt also controls differentiation. It has been shown to exhibit control over the migration of stem cell progenitors from intestinal crypts by regulating the EPH-family of adhesion molecules (Steinle *et al.* 2002). Its role in

proliferation and migration of epithelial stem cells in the normal gut has also been documented by the absence of intestinal stem cells in transgenic mice that lack the TCF4 transcription factor (Korinek *et al.* 1998). The essential nature of Wnt signalling *in vivo* is confirmed by the fact that mice lacking Wnt3a or β-catenin are not viable.

Ectopic expression of axin, an inhibitor of Wnt signalling leads to inhibition of proliferation and increased death of HSCs *in vitro* and reduced reconstitution *in vivo* (Reya *et al.* 2003). Wnt signalling can be kept in check by bone morphogenetic proteins (BMPs), which in the gut are produced by pericryptal mesenchymal cells (He *et al.* 2004): disruption of BMP signalling results in an expansion of the stem and progenitor cell compartment leading to a juvenile-polyposis-like state. BMP may be mediated by PTEN; another protein that is often mentioned as a key factor in colorectal carcinogenesis. This lipid and protein phosphatase activates BMP signalling through inhibition of the phosphatidylinositol-3 kinase (P13K)-Akt pathway, thereby preventing the nuclear localisation of β-catenin. Thus, a deficiency in PTEN in intestinal stem cells may be responsible for colorectal carcinogenesis (He *et al.* 2007).

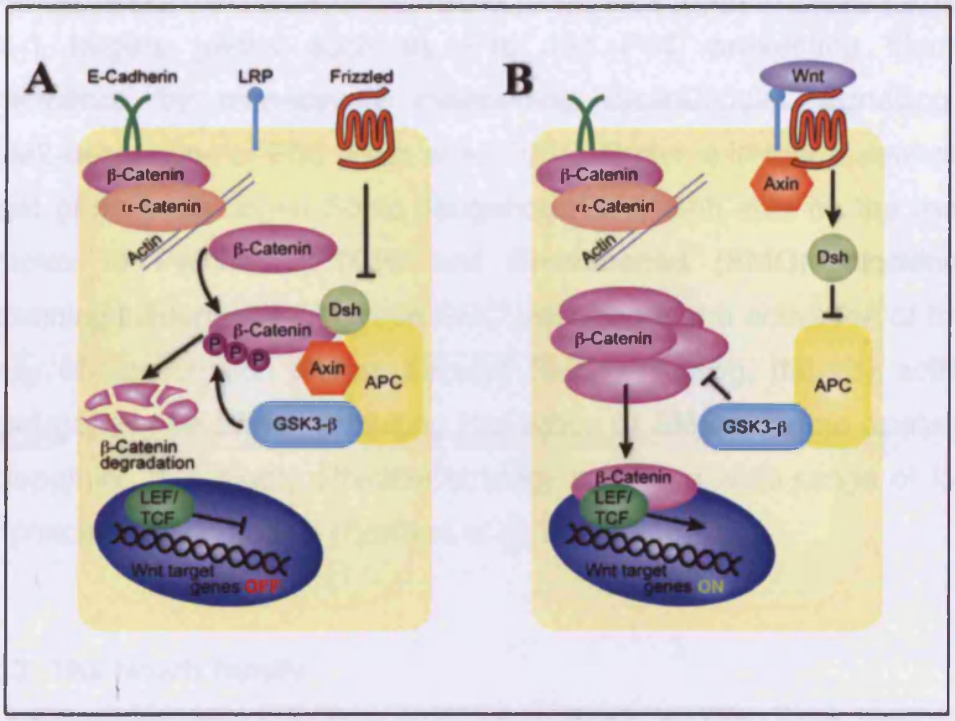

Figure 1.5. Wnt signalling pathway. (A) In the absence of Wnt signal Dishevelled (Dsh) is inactive and the destruction complex of GSK3, APC andaxin is active causing phosphorylation of β-catenin and subsequent degradation. Meanwhile, Tcf is bound to its binding site in the nucleus and represses target gene expression. (B) When a Wnt signal is present Dsh inactivates the destruction complex, allowing cytosolic accumulation of β-catenin which interacts with Tcf to allow transcription of the downstream genes. (Diagram courtesy of M.J. Lovell).

1.3.2 The Polycomb protein family

Key regulators of stem cell renewal appear to be members of the Polycomb group protein family of transcriptional repressors (Bmi-1, Rae28, Mel-18). Bmi-1 targets genes such as *P16* and *P14*, preventing stem cell senescence by respectively maintaining cyclinD/Cdk4 signalling and MDM2-destruction of P53 (Park *et al.* 2004). Bmi-1 is in fact a downstream target of the morphogen Sonic hedgehog (Shh). Shh acts on the receptor complex of Patched (PTCH) and Smoothened (SMO), blocking the restraining influence of PTCH on SMO, resulting in the activation of the Gli-family of transcription factors through SMO signalling, thereby activating target genes like *BMI-1*. Inhibiting the action of SMO with the anatagonist cyclopamine is a highly effective strategy against a wide range of foregut cancers in mouse models (Berman *et al.* 2003).

1.3.3 The Notch family

The Notch family of receptors plays a key role in intestinal stem cell physiology as well as colorectal cancer development. Triggered by binding of ligands of the Delta and Jagged families, Notch is cleaved and translocated to the nucleus where it activates the transcription factor Csl. Doing so, it controls cell self-renewal (Dontu *et al.* 2004, Karanu *et al.* 2000) and directs the differentiation of progenitor cells (Fre *et al.* 2005), thereby maintaining the balance between stem cells and differentiated cells in the intestinal epithelium. It has been shown that blocking the stem cell-maintaining activity through Notch-cleavage with γ–secretase inhibitors can reduce intestinal neoplastic development by forcing cellular differentiation (van Es *et al.* 2005).

1.4 MARKERS OF STEM CELLS

Considerable effort is being expended in the search for 'markers' of stem cells, with the expectation that many of the molecules expressed by normal stem cells will also be found in their malignant counterparts. Some proposed markers of normal human stem cells and their malignant counterparts are listed in Table 1.1 (pg.47). Collectively these molecules appear to be involved in maintaining 'stemness', such as transcription factors like Oct-4.

Many tumours appear to be caused by mutations which dysregulate normal self-renewal pathways, causing uncontrolled proliferation and resulting in neoplastic proliferation (Reya *et al.* 2001, Taipale *et al.* 2001, Reya *et al.* 2003). Thus, many of the potential stem cell/cancer stem cell markers are members of the pathways controlling stem cell self-renewal, such as proteins involved in the Wnt pathway including β-catenin, PTEN and members of the Polycomb family such as Bmi-1, PTCH. Recently, the Wnt target Lgr5 has been suggested as a new promising stem cell marker. It was shown to be expressed in mouse cycling columnar cells at the crypt base and moreover, Lgr5-expressing cells were able to generate all intestinal cell lineages, as revealed by lineage tracing experiments (Barker *et al.* 2007).

Other proteins that are commonly dysregulated in tumours are responsible for ensuring adhesion to the niche. For example, in the human epidermis, Li and Kaur have noted superior colony forming ability from cells selected on the basis of expression of the hemidesmosomal integrin α6, which partners β4 to attach cells to the basement membrane component, laminin V (Webb *et al.* 2004), while Jones and Watt noted that high expression of β1-integrin enriched for cells with colony-forming ability in basal keratinocytes (Jones *et al.* 1993).

Potential stem cell markers also include proteins that ensure their cytoprotection. CSCs undoubtedly contribute to relapsed and drug-resistant tumours because of their drug-effluxing properties. For example, haematopoietic stem cells (HSCs) have high levels of aldehyde dehydrogenase (ALDH), a detoxifying enzyme that confers resistance to alkylating agents such as cyclophosphamide (Storms *et al.* 1999). In the bronchiolar epithelium, stem cell function appears to be the property of rare pollutant-resistant cells linked to a deficiency in the phase I drug metabolising enzyme CYP450 2F2 (Giangreco *et al.* 2002, Giangreco *et al.* 2004). In the liver, progenitor cells can expand despite exposure to pyrrolizidine alkaloids because of a similar deficiency in cytochrome P450 (Vig *et al.* 2006).

1.4.1 Side Populations

Perhaps the best-known cytoprotective mechanism claimed to distinguish stem cells is conferred through the expression of members of the ABC membrane transporter superfamily. Expression of an ATP-binding cassette region enables cells to hydrolyse ATP to support energy-dependent substrate exportation against steep concentration gradients across membranes. In this manner, actively effluxing lipophilic drugs such as the fluorescent dye Hoechst 33342 causes the cells to appear as a distinct tail-shaped '*side population* (SP)' on the side of the profile when subjected to fluorescence activated cell (FAC) analysis (Goodell *et al.* 1996). Like the activity of the p-glycoprotein (encoded by the *MDR-1* gene), this activity is verapamil-sensitive, and is also sensitive to reserpine (Zhou *et al.* 2001, Zhou *et al.* 2002). Many studies suggest that the SP phenotype in rodent and human tissues is largely determined by the expression of a protein known as the ABCG2 transporter (ATP-binding cassette [ABC] subfamily G member 2, also known as breast cancer resistance protein (BCRP1), and that the SP fraction more or less equates with the stem cell population in both normal tissues (Alison 2003) and tumours (Hirschmann-Jax *et al.* 2004, Hirschmann-Jax *et al.* 2005). Moreover, not only do tumour SPs appear to have characteristics of CSCs, but additionally they may contribute to relapsed and drug-resistant cancers because of their drug-effluxing properties.

In common with many tumours, CNS tumours may have an SP fraction likely to be enriched for CSCs. They have been found in the rat C6 glioma cell line (Kondo *et al.* 2004), and in a great variety of human primary tumours (Hirschmann-Jax *et al.* 2005, Hirschmann-Jax *et al.* 2004). In two human liver tumour cell lines, a small (<1%) SP fraction has been found and transplantation of as few as 1000 of these cells was able to generate xenograft tumours in NOD/SCID mice (Chiba *et al.* 2006). Most gastrointestinal cell lines also appear to contain a small SP fraction that can self-renew, possibly being the CSCs (Haraguchi *et al.* 2006).

However, Burkert *et al.* have recently found in gastrointestinal cancer cell lines, that the SP phenotype does not enrich for cells with stem cell characteristics and thus does not represent a useful stem cell marker in these malignancies (Burkert *et al.* In Press, Chapter III).

1.4.2 Musashi, Hairy and Enhancer-of-split 1

Musashi-1 (Msi-1) is the mammalian homologue of a Drosophila protein required for asymmetric division of sensory neural precursor cells (Okabe *et al.* 2001). It is an RNA binding protein first found to be expressed in neural stem cells (Good *et al.* 1998, Sakakibara *et al.* 1996). Its expression has been confirmed in putative stem cells in intestinal crypts in neonatal and adult mice (Potten *et al.* 2003) and has been demonstrated in epithelial crypt cells of the human colon in predicted stem cell positions (Martin *et al.* 1998, Nishimura *et al.* 2003).

Hairy and Enhancer-of-split (HES)-1 is a major transcriptional repressor downstream of Notch-signalling involved in neural stem cell self-renewal and suppression of neural stem cell differentiation (Akazawa *et al.* 1992, Nakamura *et al.* 2000, Sasai *et al.* 1992). It is positively regulated by and appears to be in close association with Msi-1. It is expressed in intestinal crypt stem cell regions in the mouse small intestine, but its expression also extends to the cells migrating up the crypt (Kanemura *et al.* 2001, Kayahara *et al.* 2003). It is suggested that in the small intestine, co-expression of Msi-

1 and Hes-1 just superior to the Paneth cells is indicative of the stem cell population, whereas Hes-1 expression alone represents proliferating cells committed to differentiation (Kayahara *et al.* 2003). The significance of Msi-1 and Hes-1 as putative stem cell markers in other tissues has yet to be elucidated.

1.4.3 Surface markers

For the purpose of isolation, identification and as a potential therapeutic target, the most convenient and useful features distinguishing stem cells from somatic cells would be unique surface markers. Bonnet and Dick provided the first surface marker characterisation of CSCs. They showed that a small subset of AML cells with the same phenotype as described for primitive HSCs; $CD34^{+}CD38^{-}$ was capable of producing leukaemic progenitors and leukaemic blasts upon transplantation into immunodeficient mice, resulting in a disease phenotype identical to the donor (Bonnet *et al.* 1997, Bonnet 2005). Then in breast cancer, a subpopulation of cells with the surface antigen expression pattern $ESA^{+}CD44^{+}CD24^{-/low}$ was shown to be capable of tumour formation when implanted in limiting dilution into immunodeficient mice and of self-renewal as shown by serial transplantation (Al-Hajj *et al.* 2003). More recently, these same tumour-initiating cells have been shown capable of mammosphere generation *in vitro* (Ponti *et al.* 2005).

1.4.3.1 CD133

The most promising cell surface protein to date, implicated as stem cell marker in various tissues and cancers is CD133, also known as prominin-1 or AC133 (Shmelkov *et al.* 2005). It belongs to a family of 5-transmembrane glycoproteins, which specifically localizes to cellular protrusions. It was first shown in brain cancer that clonogenicity (neurosphere forming) and self-renewal was exclusive to the nestin and CD133-expressing subpopulation of tumour cells. In several pieces of research, the CD133-positive cell fraction isolated from different types of

brain tumours displayed stem cell properties *in vitro* and *in vivo*. *In vitro,* CD133+ cells were capable of neurosphere generation and multipotential differentiation into cells with neural and glial phenotypes, in proportions resembling the original tumour with a proliferative capacity that was proportional to the aggressiveness of the original tumour (Singh *et al.* 2003).

In vivo, intracranial injection of $CD133^+$ cells derived from various brain tumours, including the most malignant adult human CNS tumour 'glioblastoma multiforme', but also paediatric brain tumours, into NOD/SCID or nude mice at limiting dilution produced serially-transplantable tumours identical to the patient's original phenotypes. As few as 5000 cells formed tumours in nude mice, whereas injections of up to 10^5 CD133-negative cells failed to produce tumours (Hemmati *et al.* 2003, Singh *et al.* 2004, Galli *et al.* 2004, Yuan *et al.* 2004). Additionally, the CD133+ cell fraction was able to generate both CD133+ and CD133- cells, since islands of cells with both phenotypes were present within the resulting xenograft tumours. However, it is worth noting that up to 33% of cells in glioblastomas was CD133-positive, indicating that not all CD133-positive cells can be stem cells.

Recently, several reports have emerged that the CD133+ cells may also represent a stem cell-enriched population in colon cancer. CD133-positivity was found in 2.5-9% of primary human colorectal cancer cells, representing a population 200-fold enriched in serially-transplantable tumorigenic cells upon renal capsule or on subcutaneous (s.c.) injection into immunodeficient mice (O'Brien *et al.* 2007, Ricci-Vitiani *et al.* 2007).

1.4.3.2 CD44

CD44 is a widely expressed transmembrane receptor protein, and acts as an adhesion molecule in cell-cell and cell-substrate interactions. It is a proteoglycan with an NH2-terminal region, structurally related to hyaluronate binding proteins (Underhill 1992), binding to collagen and hyaluronate (Carter *et al.* 1988, Miyake *et al.* 1990). CD44 is encoded by a single gene, but exists in 10 different isoforms, generated by alternative splicing and differential glycosylation. At least in part it is involved in the Wnt signalling cascade, as its expression is activated by β-catenin (Wielenga *et al.* 1999). In normal tissues, it is involved in cell adhesion, lymphocyte homing and haematopoiesis, but it also seems to play a role in tumour progression and in the metastatic cascade, where it is thought to confer metastatic potential by changes in adhesion to extracellular ligands. Treatment of cells with an anti-CD44 variant 9 (CD44v9) and variant 6 (CD44v6) antibody has been shown to inhibit metastases in mice (Matzku *et al.* 1989, Yamaguchi *et al.* 1998).

CD44-expression differs between normal and neoplastic colonic tissue. Particularly CD44v6 and CD44v9 are differentially expressed in invasive carcinomas and carcinoma metastases, as well as in adenomatous polyps, compared to very weak expression in normal colonic epithelium (Heider *et al.* 1993). During the course of disease progression, the expression of CD44v9, normally restricted to the basal membrane, loses its polarity and increases in extent (Kawahara *et al.* 1996). CD44v6 has also been suggested to confer metastatic potential to rat tumours (Matzku *et al.* 1989).

In breast cancer, a subpopulation of cells with the surface antigen expression pattern of $ESA^{+}CD44^{+}CD24^{-/low}$ is tumorigenic and self-renewing when serially implanted into immunodeficient mice and capable of mammosphere generation *in vitro* (Al-Hajj *et al.* 2003, Ponti *et al.* 2005). Recently, the CD44-expressing population from pancreatic adenocarcinomas proved to be 4-fold enriched in tumorigenic cells, and

when CD44-expression was combined with ESA and CD24-expression, a 100-fold enrichment in tumorigenic cells was observed (Li *et al.* 2007). In various prostatic carcinoma cell lines, the CD44+ population showed a higher tumorigenicity, proliferation ability and clonogenicity than the negative counterpart. They also showed other characteristics of stem or progenitor cells, such as co-localisation with BrdU label-retaining cells, and expression of 'stemness genes' *OCT-3/4, BMI-1, β-catenin* and *SMO*. Morever, CD44+ cells generated CD44- and CD44+ cells *in vitro* and *in vivo* (Patrawala *et al.* 2006). Another recent study has shown that CD44+ cells from head and neck squamous cell carcinomas (SCC) have the ability to self-renew and differentiate *in vivo*, have a primitive morphology, and co-stain with the basal stem cell marker cytokeratin 5/14 and Bmi-1 (Prince *et al.* 2007). During the course of our experiments (chapter IV), a report emerged identifying the CD44+/EpCAM^{high} population as a tumorigenic cell population in colorectal cancers when s.c injected into NOD/SCID mice (Dalerba *et al.* 2007).

1.4.3.3 CD117

CD117 is a 145 kD tyrosine kinase receptor encoded by the proto-oncogene *c-KIT*, acting as a receptor for the cytokine stem cell factor (SCF). Dimer formation with its ligand causes indirect activation of several signalling cascades that are involved in cell survival, adhesion, proliferation, and differentiation (Kitamura *et al.* 1998, Alexander *et al.* 1998, Ashman *et al.* 1999). The fact that animals deficient in CD117 fail to develop highlights its importance for cell survival and differentiation (Alexander *et al.* 1991). CD117 was first demonstrated differentially expressed on the surface of haematopoietic stem cells (HSCs), multipotent progenitors and myeloid progenitors. Since then, its expression has also been observed on thymocyte progenitors (Massa *et al.* 2006), mast cells, melanocytes in the skin, and interstitial cells of Cajal (Bernex *et al.* 1996) in the digestive tract. Another target of CD117-expression appears to be hair matrix keratinocytes of melanocyte-deficient hair follicles in mice treated with an anti-Kit antibody, and was found to be hair-cycle-dependent. Involvement in

hair growth control was also indicated by the observation that anagen development in c-KIT-deficient mice is significantly retardxed (Peters *et al.* 2003).

Differential overexpression of CD117 in many malignant tissues suggests roles in growth, survival, migration, and invasive potential of malignant cells. Acting as a proto-oncogene, activation mutations in the *c-KIT* sequence can cause its overexpression, contributing to tumour development by its effect on signalling cascades involved in cell cycle-regulation (Turner *et al.* 1992).

Overexpression of *c-KIT* has mainly been associated with lymphoproliferative disease, but it has also been demonstrated in various non-haematopoietic malignancies including gastrointestinal stromal tumours (Druker 2001, Kitamura *et al.* 1998), mast cell disease (Heinrich *et al.* 2002), neuroblastoma (Timeus *et al.* 1997), malignant cells from breast (Hines *et al.* 1999), lung (Turner *et al.* 1992) and prostate (Simak *et al.* 2000). Immunohistochemical studies have also suggested an involvement of CD117-expression in endometrial carcinogenesis (Elmore *et al.* 2001). The effect of CD117 was in many instances achieved through inhibition of apoptosis, which has been shown in melanocyte precursors (Ito *et al.* 1999), soft tissue sarcomas (Ricotti *et al.* 1998), neuroblastomas (Turner *et al.* 1992), and normal and malignant haematopoietic cells (Hassan *et al.* 1996).

The extent and involvement of CD117-expression in colorectal carcinomas is unclear. In human carcinoma cell lines, such as HT-29, overactivation of *c-KIT* was responsible for anchorage-independent growth (Bellone *et al.* 2001, Lahm *et al.* 1995). However, other studies on primary tissues observed CD117 expression as a rare event, only seen in 1.6% of 126 colorectal carcinoma specimens and altogether absent from normal colon (Reed *et al.* 2002). Together, the expression of CD117 on several types of stem cells and differential expression in tumours renders it a possible candidate as a potential cancer stem cell marker.

However, contrasting evidence argues that *c-KIT* activation does not play a universal role in stem cell function and cell survival. CD117 is not ubiquitously expressed on malignant or metastatic cells. In advanced melanoma cells for example, its expression appears to be very low (Montone *et al.* 1997). Forced *c-KIT* expression inhibits the metastatic potential of melanoma cells through SCF-induced apoptosis; a mechanism to be explored as a novel therapeutic approach (Huang *et al.* 1996).

1.4.3.4 CD34

CD34 is a sialomucin-like adhesion molecule that is specifically expressed on the cell surface of human haematopoietic stem cells (HSC) (Lubbert *et al.* 1991) and has been implied as a potential stem cell marker in several non-haematopoietic tissues. Encoded on gene locus 1q32, its gene product is a 100 kD transmembrane protein, the function of which has not been elucidated to date (Satterthwaite *et al.* 1992). First demonstrated in baboons, it was shown that the CD34-positive population of primate BM shows a high efficiency in clone formation and is capable of repopulating lethally-irradiated BM *in vivo* (Berenson *et al.* 1988, Civin *et al.* 1984). This fact is the basis for the widely used clinical application of CD34 in selecting haematopoietic stem cell progenitors for BM transplantation (Sutherland *et al.* 1993). Its expression has also often been implicated in haematopoietic malignancies. However, CD34-expression in tumours seems variable, possibly having a prognostic significance (Stagno *et al.* 1996).

On the other hand, SP cells from human BM, known to restore BM function in lethally irradiated mice, are in many tissues CD34-negative (CD34-), suggesting that a CD34- HSC population exists (Goodell *et al.* 1997). This was also confirmed by other researchers who demonstrated that CD34- cells show stem cell-typical behaviour such as multilineage haematopoiesis, as well as the ability to generate CD34-positive (CD34+) cells *in vitro* and *in vivo* (Zanjani *et al.* 1998). Based on the bulk of conflicting evidence, a mechanism has been suggested whereby human HSCs possess reversible CD34-expression ability with the CD34- HSC

population representing a more primitive SC population and the CD34+ cell population being enriched in more rapidly-proliferating progenitor cells.

A similar *scenario* has been proposed in mice. HSCs are found in both the CD34+ and CD34- cell fractions. CD34-deficient mice show a delay in haematopoietic development. However, in the blood of adult mice, the haematopoietic profile is normal, suggesting, that CD34-expressing cells, although not essential for survival, are involved in mouse haematopoiesis (Cheng *et al.* 1996). On the other hand, transplantation of CD34- cells can result in haematopoietic reconstitution and multilineage differentiation, implying their stem cell potential (Osawa *et al.* 1996). A mechanism has been suggested whereby CD34 expression on murine HSCs is reversible: CD34- cells start expressing CD34 when stimulated by early-acting cytokines, but still retaining their HSC function. When cell activation is terminated, they presumably revert back and stop CD34-expression (Sato *et al.* 1999, Ito *et al.* 2000, Matsuoka *et al.* 2001).

CD34 has also been associated with stem cells from other tissues. In the portal vein endothelium of the liver, it is co-expressed with the liver stem cell marker Thy-1 (Terrace *et al.* 2007). In the hair follicle, CD34-expression is specifically targeted to a known stem cell area, the hair follicle bulge, in several species, including humans, mice and rabbits (Pascucci *et al.* 2006, Poblet *et al.* 2006, Raposio *et al.* 2007). Within the bulge, CD34-expression was co-localised with expression of the stem cell marker keratin 15 in mouse hair follicles (Poblet *et al.* 2006) and with the proliferation marker Ki-67 in humans (Raposio *et al.* 2007). Additionally, its expression is implicated in the progression (Affara *et al.* 2006) and may even be essential for the formation of skin tumours (Trempus *et al.* 2007).

Table 1.1

Some proposed markers of normal human stem cells and their malignant counterparts

Tissue/disease	Normal tissue stem cells	Cancer stem cells
Brain/medulloblastoma	CD133+nestin+GFAP+ A2B5+ Musashi1 SSEA-1	$CD133^{+}$
Breast	$a6^{+}CK19^{+}ESA^{+}MUC1^{-}$ CALLA-	$ESA^{+}CD44^{+}CD24^{-/low}$
Haematopoietic/CML	$CD34^{+}CD38^{-}$ $CD133^{+}$ $ALDH^{+}$ SP	$CD34^{+}CD38^{-}$
Gastrointestinal	Musashi1 Hes-1 Nuclear β-catenin	SP – found in cell lines

1.5 CLONALITY

A major difficulty in finding methods to isolate and identify stem cells, which prevents them from being identifiable and examined directly in many tissues is their immature phenotype. To circumvent this obstacle, researchers rely on the fact that stem cells give rise to clonal populations of differentiated and identifiable progeny. Investigating the progeny can indirectly reveal information about normal and pathological developments within their ancestors. Additionally, the difficulty in studying developmental dynamics *in vivo* renders following clonality and the spread of mutations the method of choice to provide insight into tissue development. Patterns of mutations that do not confer a selective advantage or disadvantage allow for the study of tissue and tumour development.

1.5.1 Genomic variations as markers of clonality

Mosaic animals and mouse aggregation chimeras with somatic mutations in the *Dlb-1* locus on chromosome 1, affecting the lectin-binding ability for *Dolichos biflorus* agglutinin (DBA), have been used to follow clonality and tissue development in the intestinal tract. The lectin-binding ability can be lost by spontaneous mutation of the *Dlb-1* locus, or by treatment with the chemical mutagen ethylnitrosourea (ENU). In heterozygous mouse aggregation chimeras derived from a cross between a C57Bl/6J wild type strain and the SWR strain containing a carbohydrate polymorphism of *Dlb-1*, DBA can only bind to clones of C57Bl/6J-derived, but not to SWR-derived cells (Schmidt *et al.* 1988). Similarly, ENU treatment results in loss of DBA binding in mutated cells, and if the mutation of the *Dlb-1* locus happens in a stem cell, it then expands stochastically to produce a clone of cells that cannot bind DBA (Winton *et al.* 1988).

Mutations and known polymorphisms of the X-linked gene glucose-6-phosphate dehydrogenase (*G6PD*) have also been exploited for clonality studies revealing the mosaic patterns of *G6PD* staining in heterozygous

animals. Use of this model to investigate clonality excludes the possibility that cells derived from distinct strains in chimeric mice segregate differentially during organogenesis (Thomas *et al.* 1988). In humans, a convenient method for studying clonality is to follow X-inactivation patterns or X-linked heterozygous polymorphisms, since it is well known that X-inactivation is inherited to daughter cells in a clone (Novelli *et al.* 2003). One study that provided important information regarding the clonal origin of human intestinal crypts came from the examination of the colon of a rare XO/XY patient who had received a prophylactic colectomy for familial adenomatous polyposis (FAP) (Novelli *et al.* 1996). However, studies involving X-linked mutations and inactivation patterns, although convenient and easily carried out, can only be carried out on females and exclude male development and potential sex-specific developmental differences from the research.

As a solution, examining methylation patterns can make it possible to map stem cell division histories and cell fate by following the methylation pattern of non-expressed genes (Yatabe *et al.* 2001, Kim *et al.* 2002).

1.5.2 Mitochondrial mutations as markers of clonality

Mitochondria (mt) are cellular power generators, creating cellular ATP through oxidative phosphorylation. They contain a circular 16.6 kb DNA molecule encoding 37 genes, of which 22 encode tRNA, 2 rRNA, and 13 proteins involved in oxidative phosphorylation. Each mitochondrion contains several hundred molecules of its circular genome and in most cells several thousand of these organelles exist. Mt contain their own replication machinery and genetic code and lack protective histones and DNA repair mechanisms which renders the rate of mutation random; higher than in genomic DNA and increasing with age. These properties, in addition to their abundance, make them important tools in forensic science, specifically useful in heavily-degraded, aged and small samples. Mt DNA mutations can produce a biochemical deficiency dependent on the site and severity of the mutation and have been implicated in the progression of many

diseases, including carcinogenesis (Brierley *et al.* 1998, Michikawa *et al.* 1999, Coller *et al.* 2001, Taylor *et al.* 2003). Mt mutations can spread throughout the cells through mt replication and render the cells *heteroplasmic* with a mixed population of mutated and wt mitochondria, or *homoplastic* with all mitochondria in a cell mutated or wt. For a cell to show an overall mutated mt phenotype, homoplasmy or low levels of heteroplasmy must be present.

Non-oncogenic mitochondrial DNA (mtDNA) mutations, which result in the deficiency of cytochrome c oxidase, have been used as a marker of clonality in the intestinal tract, and provided information regarding SC numbers and maintenance dynamics in the normal and dysplastic colon and stomach (Greaves *et al.* 2006, McDonald *et al.* 2006). Cells that demonstrate a deficiency in cytochrome c oxidase function and normal succinate dehydrogenase (SDH) activity generally have increased numbers of overall mtDNA mutations (Johnson *et al.* 1993). Cytochrome c oxidase is a large lipoprotein composed of 13 subunits. In mammals, 10 subunits are encoded by the nuclear genome and 3 are synthesized mitochondrially. It is the last protein in the electron transport chain and catalyses the reduction of dioxygen to water, thereby generating an electro-chemical gradient that provides cellular energy. In a histochemical method to detect the activity of cytochrome c oxidase, cells with mutations affecting cytochrome oxidase activity stain blue, whereas normal cells stain brown.

1.6 THE INTESTINAL EPITHELIUM

1.6.1 Cell lineages of the intestinal epithelium

The intestine consists of the small and large intestine, both of which are composed of three cell layers: the lamina propria and the submucosa are the two outermost layers and serve as structural and regulatory support. The vascular lamina propria is composed of numerous cell types such as fibroblasts, fibrocytes, vascular endothelial and smooth muscle cells, blood cell lineages and intestinal subepithelial myofibroblasts (ISEMF). ISEMFs

secrete growth factors, such as transforming growth factor beta 2 (TGFβ2) and Wnt, which is known to play a role in stem cell differentiation and proliferation (Powell *et al.* 1999). The innermost layer consists of columnar epithelial mucosa with glandular invaginations, the crypts of Lieberkuhn. As concave indentations, the crypts extend through the lamina propria down to the muscularis mucosae. In the small intestine, crypts give rise to fingerlike projections known as villi, whereas the colon is devoid of villi. Cells are organised in a hierarchical migratory fashion within the crypts and villi, with fully-differentiated cells towards the luminal surface, which are continually shed into the lumen and replaced by a constant stream of proliferating progenitor cells located in the bottom two-thirds of the crypt.

There are four common cell lineages contributing to the epithelial layer in the small intestine, all of which are reconstituted by the same stem cell population. Absorptive and columnar cells are the predominant cell type, known as 'enterocytes' in the small intestine and 'colonocytes' in the colon. Columnar cells are polarised cells with a basal nucleus and an apical brush border composed of microvilli, which increase the surface area. The gastrointestinal tract is the largest endocrine organ in the body, a function, which is provided by an abundance of 'endocrine', 'neuroendocrine' or 'enteroendocrine' cells that secrete peptide hormones. Approximately 10 different endocrine cells, defined by their hormonal content are known to date in the mammalian small intestine (Solcia *et al.* 1998). Goblet cells are dispersed throughout the epithelium and secrete mucin granules to lubricate the mucosa and expel microorganisms. Paneth cells are the only exception to the polarized hierarchical structure of the epithelial cell layer. They are the only cells in the small intestine that escape the upwards-migratory flow of differentiating cells. All other cell types migrate upwards along the crypt-to-villus axis as they become more differentiated and undergo apoptosis, with subsequent shedding when they reach the intestinal lumen, but Paneth cells migrate downwards during maturation (Bjerknes *et al.* 1981). They secrete proteins that keep the crypt sterile, such as lysozyme, tumor necrosis factor α and antibacterial defensins or

cryptdins, from large apical secretory granules (Darmoul *et al.* 1997, Ouellette *et al.* 1994, Ouellette *et al.* 2000, Ouellette 2004).

Other less common cell lineages are also present, such as caveolated cells (Nabeyama *et al.* 1974), and M- (membranous- or microfold-) cells that are involved in antigen transport and reside in the small intestine near specialised lymphoid follicles, known as 'Peyer's patches' (Gebert *et al.* 1997) (reviewed in (Kerneis *et al.* 1999)).

1.6.2 Developmental dynamics in the intestinal epithelium

It is believed that crypts are polyclonal at birth, but subsequently convert to pure monoclonal crypt populations. This has been shown in aggregation chimeric mice with *Dlb-1* mutations (Schmidt *et al.* 1988). When treated with mutagens, monoclonal conversion in mouse small intestinal crypts takes 8-12 weeks, whereas in the colon, it only takes 4 weeks. The temporal differences in the appearance of monophenotypical crypts may be due to differential rates of crypt fission at the time of mutagen administration (Park *et al.* 1995), but it could also reflect different stem cell numbers. After irradiation, human homozygotes for mutations in the O-acetyl transferase gene suggested a monoclonal conversion time of one year in human colonic crypts (Campbell *et al.* 1996).

Crypts divide and expand in number by crypt fission, whereby crypts divide longitudinally after basal bifurcation resulting in two daughter crypts (Park *et al.* 1995, Greaves *et al.* 2006). Clonal patches of crypts and observations of bifurcating crypts in steady state provide evidence for this mechanism. In humans, the crypt cycle takes approximately 9-18 years, and in mice 108 days (Bjerknes 1986).

The mechanisms that stimulate a crypt to divide are unknown. It has been suggested that high crypt fission rates are linked with increased stem cell numbers (Totafurno *et al.* 1987). This theory however, is challenged by observations of increased numbers of bifurcating crypts upon chemical

mutagenesis (Loeffler *et al.* 1993). Another model suggests that crypt fission is initiated upon crypts reaching a certain threshold size (Park *et al.* 1995).

No matter what the mechanisms of fission initiation are, fission itself results in the formation of patches of related crypts. Novelli *et al.* utilised the heterozygosity for a mutation in the X-linked *G6PD* gene (563 C>T) present in 17% of Sardinian females to analyse patch size by histochemical staining for G6PD. They have shown that crypts exist in large families of either G6PD-positive or negative crypts. Of 10,538 colonic crypts analysed from 9 patients, patch size in the colon was found to comprise up to 450 crypts (Novelli *et al.* 2003). Crypt fission is increased in pre-neoplastic lesions such as aberrant crypt foci (ACF). On this view, crypt fission is a vital component of the ACF-adenoma-carcinoma pathway and seems to be the main mechanism of the extra-cryptal spread of clones containing mutations in the adenomatous polyposis coli (*APC*) gene, frequently mutated in colorectal cancer (Kinzler *et al.* 1996).

1.6.3 Intestinal stem cells

The intestinal mucosa undergoes constant and rapid cell turnover with a frequency of every 2-3 days in rodents and other mammals (Goodlad *et al.* 1984). The high regenerative pressure is managed by careful homeostasis between cell proliferation, differentiation, senescence and apoptosis, governed by the stem cells. In spite of their immense importance for intestinal function, limited and controversial evidence exists regarding the location, quantity, regulatory pathways or function of intestinal stem cells. This is exacerbated by the fact that to date there is no culture method robust enough to allow primary culture of the intestinal epithelium to test proliferative and differentiation potential of intestinal cellular subtypes.

Ablation studies of specific epithelial cell lineages have failed to identify candidate stem cells. Targeted ablation of Paneth cells (Garabedian *et al.* 1997), and three different endocrine cell lineages in the mouse small

intestine (Rindi *et al.* 1999), did not affect epithelial cell proliferation. It is thus conceivable that the stromal/mesenchymal cells comprising the stem cell niche set up an optimal microenvironment through their secretion of specific growth factors and inflammatory cytokines, and play an important role in stem cell function, perhaps more so than the genetic programming within the stem cells themselves (Dignass *et al.* 1994, Riegler *et al.* 1996, Podolsky 1997, Polk 1998, reviewed in (Powell *et al.* 1999), Dignass 2001).

1.6.3.1 Stem cell location

In the small intestine, the stem cell niche is believed to reside in position 4-5 counted from the bottom of the crypt with positions 1-3 occupied by Paneth cells (Figure 1.6, pg.55). In the colon, where Paneth cells are absent, the niche is believed to lie directly at the crypt base (Alison *et al.* 2000). In the mouse small intestine, these stem cell positions were proposed in the 'stem cell zone' hypothesis. Following a series of microscopic morphometry and autoradiography-labelling studies, it was suggested that 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 (Bjerknes *et al.* 1981, I-V). This is consistent with radiation experiments, which gave rise to a hierarchical crypt model whereby cells move through three tiers upon leaving the stem cell compartment, continuing to divide, but gradually losing their clonogenic ability. Some cells within the three tiers may be capable of reconstituting a crypt in the case of damage (Marshman *et al.* 2002). This view however, is disputed by some researchers following recent observations (Barker and Clevers, 2007).

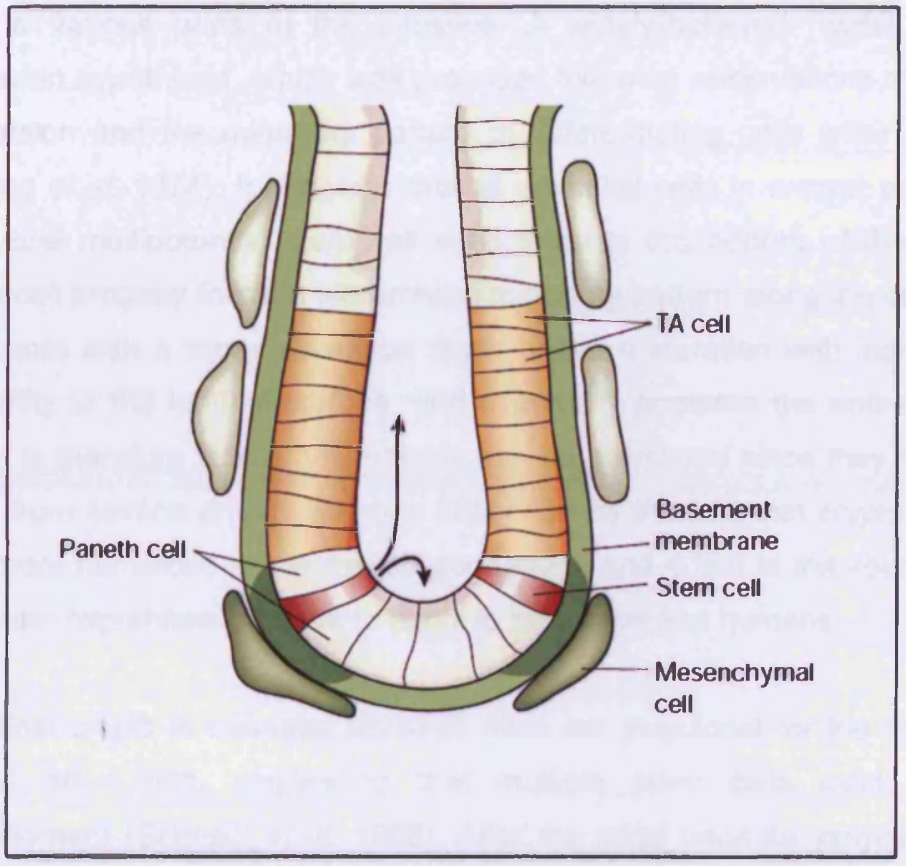

Figure 1.6. The human small intestinal crypt. Stem cells are believed to be located at position 4-5 from the bottom, just above the Paneth cells. Differentiating cells migrate upwards out of the stem cell zone towards the luminal surface, first as transit amplifying cells (TAC), which then proceed to terminal differentiation. Paneth cells are an exemption and migrate downwards from the stem cell zone. (Figure taken from Spradling *et al.*, 2001)

1.6.3.2 Stem cell number

Much controversy exists regarding the stem cell numbers in the intestine and in various parts of the intestine. A widely-believed model is the 'Unitarian hypothesis', which was proposed following observations of clonal expansion and the migratory pattern of differentiating cells within crypts (Cheng *et al.* 1974). It suggests that all epithelial cells in a crypt originate from one multipotential stem cell sited towards the bottom of the crypt. Stem cell progeny follow a hierarchical migratory pattern along the crypt-to-villus axis with a more advanced stage of cell maturation with increasing proximity to the luminal surface, and eventually populate the entire crypt, which is therefore clonal. Villi notably can be polyclonal since they receive input from several crypts, which is supported by the fact that crypts are 7-fold more numerous in the mouse duodenum, and 4-fold in the ileum. The Unitarian hypothesis appears to apply to both mice and humans.

Intestinal crypts in neonatal B6/SWR mice are polyclonal for the first two weeks after birth, suggesting that multiple stem cells exist during development (Schmidt *et al.* 1988). After the initial neonatal polyclonality, the epithelial cells in the crypts become monoclonal in the adult mouse intestine, possibly due to positive selection of a single dominant clone or by segregation of lineages due to the division of crypts by 'crypt fission', which occurs frequently during this developmental period. These observations infer the existence of one sustaining stem cell (Ponder *et al.* 1985, Winton *et al.* 1990, Park *et al.* 1995, Bjerknes *et al.* 1999). Radiation studies, where eradication of all but single clonogenic cells resulted in the repopulation of all cell types within the mouse crypt, also support this view (Withers *et al.* 1970). Similarly, ENU-triggered mutation of the *Dlb-1* locus in a stem cell subsequently led to stochastic cell expansion to produce a clone of cells which contained all epithelial lineages within the small intestinal crypt (Winton *et al.* 1988). Temporal differences in the time taken for mutant stem cells to expand stochastically and create monoclonal crypts led to the suggestion that larger numbers of stem cells are present in the colon than in the small intestine (Williams *et al.* 1992).

In humans, a fortuitous discovery by Novelli and colleagues in 1996 revealed that crypts were clonal. In a patient with familial adenomatous polyposis (FAP), who had undergone prophylactic colectomy and was also chimeric for the Y chromosome (each cell either XO or XY), crypts were either positive or negative for the Y chromosome, but rarely mixed (Novelli *et al.* 1996). Only 4 of 12,614 crypts examined showed a mixture of XO and XY cells, which was explained by non-disjunction with loss of the Y chromosome in a crypt stem cell. However, the small intestinal villi were generally mixtures of XO and XY cells, consistent with the notion that villi are fed from more than one crypt. Clonality must be determined at the patch edge, since cells within the centre of a patch could be truly clonal or simply monophenotypic, formed from several stem cells of the same genotype. Examination of 2,260 crypts located at the patch periphery of females with heterozygosity for the *G6PD* Mediterranean mutation also showed no evidence of any crypts with a mixed phenotype, indicating that colonic crypts must be monoclonally-derived (Novelli *et al.* 2003).

Further to this, other studies have shown that single cell cloned HRA-19 cell lines can induce tumours in mice that contain all the major differentiated intestinal epithelial cell lineages (Kirkland *et al.* 1994) – strong evidence supporting the Unitarian hypothesis, although this cell line was derived from a colonic adenocarcinoma which should not automatically be assumed exemplary for the same mechanisms in the normal epithelium.

However, recent important research has proposed that crypts contain multiple stem cells, revealed by ribbons of cells reaching from the crypt bottom to the top that showed the same mutation in the mitochondrially-encoded cytochrome c oxidase gene in amongst cells that are otherwise cytochrome c oxidase-positive (Taylor *et al.* 2003).

Additionally, phylogenetic analyses of methylation patterns of three neutral loci show variations in cells in separate, unrelated crypts, whereas cells within one crypt display closely related or identical methylation patterns (Yatabe *et al.* 2001). This suggests that human colonic crypts contain

multiple stem cells, which are constantly lost and replaced, eventually leaving all cells monoclonally-derived, related to the closest ancestor; a process called 'monoclonal conversion'

Attempts to determine the exact number of stem cells within each crypt have yielded variable results. Estimates based on radiaton experiments range from 0.4-60% of all crypt cells, with species-specific differences (Marshman *et al.* 2002). Cell kinetic and mathematical simulation data suggest that by clonal expansion, 4-6 stem cells per crypt could easily maintain the mammalian intestinal epithelium. This number emerged when epithelial cells were randomly marked by *DBA* or *G6PD* mutations and followed with regards to position, morphology and longevity (Bjerknes *et al.* 1999, Griffiths *et al.* 1988). Other reports have also determined 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 *et al.* 1997, Potten 1998,)

However, others claim that up to 16 or more stem cells can exist in a single intestinal crypt (Roberts *et al.* 1995). It has also been 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).

1.6.4 Intestinal cancer

Cancer of the intestinal tract has an estimated lifetime risk of 5% (Horne-Badovinac *et al.* 2003). This high frequency is attributable to the constant cell turnover and necessary stem cell proliferation within the intestinal epithelium and the comparatively high exposure to carcinogens throughout the intestinal tract. Although intestinal cancer is among the best-understood solid malignancies on a molecular and genetic level, little is still known regarding the cellular origins of gastrointestinal malignancies.

There are two conflicting models of intestinal neoplastic development in humans, both regarding stem cells as their origin. According to the 'bottom-up' theory, stem cells acquire the carcinogenic mutation within their niche at the crypt base and subsequently expand to colonise the entire crypt with their neoplastic progeny (Preston *et al.* 2003). This is followed by increased crypt fission giving rise to an oligocryptal adenoma and further leading down the adenoma-carcinoma path. This model is in accordance with the notion that intestinal stem cells are located near the crypt base. By contrast, the 'top-down' hypothesis is based on empirical evidence of dysplastic cells exclusively located at the orifices and luminal surface of colonic crypts (Shih *et al.* 2001). Since there is no evidence for a stem cell population in the intracryptal zone, the original top-down hypothesis, whereby transformed intracryptal stem cells migrate laterally and downwards into the crypt has been modified. It is now proposed that after stem cells acquire a second hit at the crypt base, the daughter cells undergo neoplastic expansion upon reaching the intracryptal zone. There is evidence for both mechanisms of adenoma morphogenesis and thus, both of these mechanisms may hold true. Whereas top-down spread seems to occur more in advanced adenomas, in early adenomas, only bottom-up development can be observed.

Defects in the adenomatous polyposis coli (*APC*) gene, which plays an important role in regulating the Wnt pathway through β-catenin degradation, are the most commonly found genetic abnormalities in colorectal tumours. Additionally, it regulates stem cell division by establishing correct cell polarity through indirect interactions with microtubules (Mogensen *et al.* 2002, Yamashita *et al.* 2003). Misalignment of the mitotic spindle following loss of *APC* function may lead to dysregulated stem cell division and increased cell proliferation. Hundreds of mutations have been identified within this gene, many of which with specific prognostic implications. *APC* has the first genetic alterations in the development of gastrointestinal neoplasia according to the adenoma-carcinoma sequence of events (Morson 1974) as most patients with hereditary Familial Adenomatous Polyposis (FAP) carry a germline mutation of the *APC* gene (Bodmer *et al.*

1987). A second mutation in the other allele and thus loss of function of the *APC* tumour suppressor results in development of a monocryptal adenoma which may further progress into microadenoma, macroscopic adenoma and finally colorectal carcinoma (Knudson 1993, Miyaki *et al.* 1994).

1.7 Skin

The skin is the largest organ in the human body, providing a protective barrier to environment. Its outer layer, the epidermis, is composed of a multilayered stratified squamous keratinised epithelium, the interfollicular epidermis (IFE), interspersed with hair follicles, sebaceous and sweat glands (Figure 1.7, pg.61). Cells are continuously shed from the epidermal surface and homeostasis is maintained by basally situated self-renewing stem cells (SC), which generate daughter cells capable of terminal differentiation into the various epidermal cell lineages.

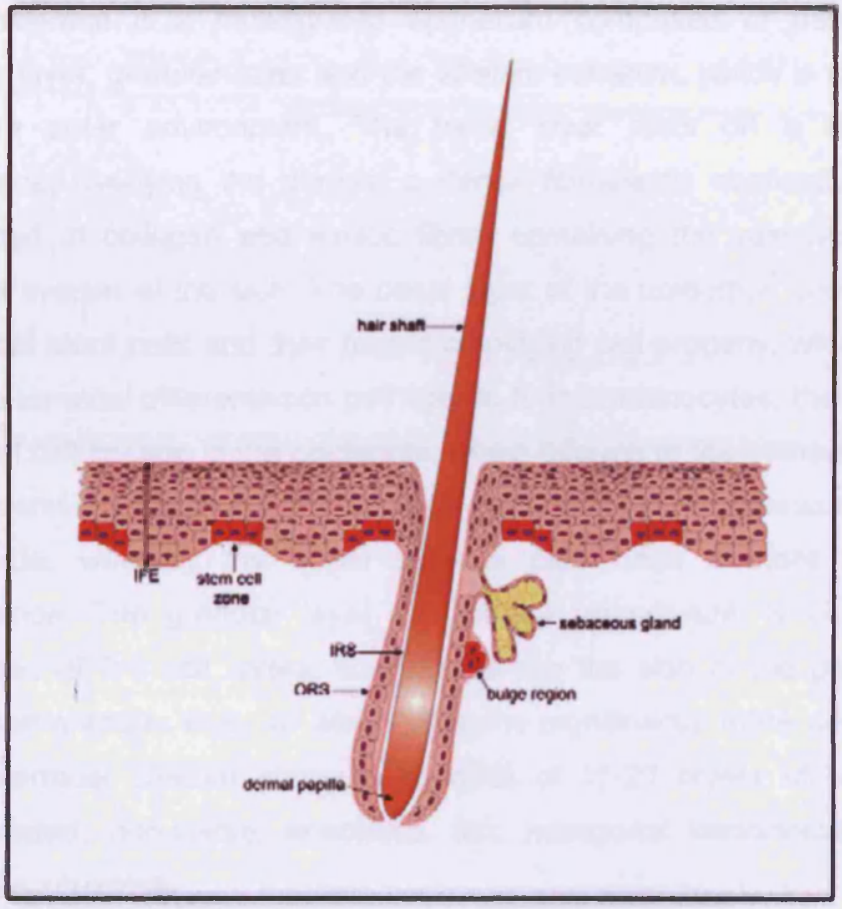

Figure 1.7. The epidermal structures with putative associated stem cell zones. The stem cells of the interfollicular epidermis are believed to be at the top of the rete ridges (basal invaginations). The bulge region is a postulated stem cell zone in the hair follicle. The outer root sheath (ORS) together with the inner root sheath (IRS) line the hair shaft. (Figure courtesy of M.J. Lovell).

1.7.1 The interfollicular epidermis

1.7.1.1 Architecture of the interfollicular epidermis

The epidermis is a multilayered epithelium composed of basal layer, spinous layer, granular layer and the *stratum corneum*, which is in contact with the outer environment. The basal layer rests on a basement membrane, overlying the dermis, a dense fibroelastic connective tissue composed of collagen and elastic fibres containing the vasculature and nervous system of the skin. The basal layer of the epidermis contains the epidermal stem cells and their transit amplifying cell progeny, which follow a single terminal differentiation pathway to form keratinocytes, the principal epithelial cell lineage in the epidermis, which migrate to the corneal layer of the epidermis. In humans, the spinous layer, or *stratum spinosum*, is 4-8 cells wide, whereby the upper spinous cells have a more flattened appearance. The granular layer, or *stratum granulosum*, is in humans comprised of 2-3 cell layers. Exemptions are the skin of the palms and soles, the granular layer of which contains significantly more cell layers. The outermost *stratum corneum* consists of 15-20 layers of terminally differentiated, non-viable, anucleate, flat, hexagonal keratinocytes, also known as squames.

1.7.1.2 Cell lineages of the interfollicular epidermis

Keratinocytes are the principal epithelial cell type in the epidermis. Their cytoskeleton is mostly composed of keratins, a family of filamentous proteins and differs slightly in structure depending on differentiation stage and layer position. The differentiation cycle of keratinocytes is approximately 8-9.5 days in mice (Potten *et al.* 1987), and between 28-60 days in humans (Hunter *et al.* 1995). The intracellular cytoskeleton of basal cells is composed of a dispersed, extensive network of keratin filaments. The spinous layer contains post-mitotic keratinocytes with large, dense bundles of keratin intermediate filaments extending to the cell periphery to form desmosomal cell junctions, which equip the tissue with mechanical strength. Spinous keratinocytes also express lamellar keratohyalin

granules, which secrete lipids and enzymes that fill the intercellular spaces of the *stratum corneum*, thereby contributing to the barrier function of the epidermis. The keratinocytes in the *stratum corneum* are fully terminally differentiated, devoid of a nucleus, not viable and are eventually shed from the epidermal surface. The cornified cell envelope of the *stratum corneum* consists of crosslinked structural proteins synthesised by granular keratinocytes.

Non-keratinocyte cell lineages in the epidermis comprise melanocytes, Langerhans and Merkel cells, which together account for less than 20% of all epidermal cells. Langerhans cells are found suprabasally in the spinous layer. They provide immunological protection through uptake, processing and presentation of specific antigens. Distinguished by a dendritic appearance, Langerhans cells lack keratin filaments, but possess 'Birbeck granules', which enclose bound antigen through the formation of invaginations in the plasma membrane (Takahashi *et al.* 1985).

The basally-situated melanocytes function in their absorption of ultraviolet light. They are also of a dendritic phenotype, lack keratin filaments and are distinguished by the presence of small, membrane-bound melanin pigment-containing melanosomes. Via the dendritic processes, melanin is transferred to keratinocytes. Melanocytes and keratinocytes occur in a precise 1:36 ratio, forming a regular structural element known as the 'epidermal melanin unit' (Fitzpatrick *et al.* 1963, Jimbow *et al.* 1976).

Merkel cells are hormone-secreting cells which function as slow-adapting mechanoreceptors of the epidermis (Halata *et al.* 2003). In contrast to the non-keratinocyte cell types, Merkel cells can synthesise keratin intermediate filaments in their small, membrane-bound dense core granules. However, the keratin filaments are loosely distributed within the cytoplasm and do not occur in structurally organised bundles as it is the case in keratinocytes. Merkel cells are the least common non-keratinocyte lineage in the epidermis, with elevated numbers in regions of high touch

sensitivity such as palms, buccal mucosa, lips and soles (Lacour *et al.* 1991).

1.7.2 The sebaceous gland

The sebaceous glands are attached to the upper permanent portion of the hair follicle where they operate independently of the hair cycle. With the exemption of hairless body areas such as palms and soles, sebaceous glands are always associated with hair follicles. The greatest density of glands is found on the face and scalp. The glands vary in size and can be composed of one or multiple lobules, independent of their location.

Cells of the sebaceous glands follow a unidirectional differentiation pathway featuring a single cell lineage, the sebocytes. As they differentiate, the cells migrate from the basal layer toward the centre of the gland, gradually accumulating a lipid-rich fluid, or sebum. At the sebaceous duct their sebum content becomes so high that nuclei and other intracellular structures disappear. The cells eventually rupture and release their contents via the duct by holocrine secretion (reviewed in (Thiboutot 2004)).

1.7.3 The hair follicle

Hair follicles are more complex and comprise eight different cell lineages that form an epithelial core surrounded by a mesenchymal sheath. The hair shaft is composed of the medullary cells in the centre, surrounded by cortical and hair cuticle cells. 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. Surrounding the dermal papilla is a matrix region, which contains progenitor cells that give rise to the hair shaft (reviewed in (Goldsmith 1991)).

The hair shaft is enclosed within the inner root sheath (IRS), composed of three cell lineages, namely from centre to periphery: cuticle, Henle layer and Huxley's layer. The outermost layer of the hair follicle is the outer root

sheath (ORS), continuous with the overlying interfollicular epidermis (IFE). The ORS is believed to harbour a stem cell population and play a special role in the maintenance of the multiple hair lineages (reviewed in (Watt 2002, Watt *et al.* 2006). The IRS and ORS are separated by the companion layer, the evolutionary origin of which and its attribution to either the IRS or the ORS is subject to debate. Analysis of keratin expression patterns ascribed it to the IRS as its outermost layer (Gu *et al.* 2007).

Hair follicles undergo a constant cycle of alternating growth (anagen), regression (catagen) and rest (telogen). In the mouse, anagen lasts approximately three weeks. In humans, anagen varies depending on body region. In the scalp, the anagen phase of hair follicles can last several years, whereas in hair follicles of eyelashes and eyebrows it lasts around 1-4 months. Each follicle maintains an independent rhythm of growth and rest (reviewed in (Alonso *et al.* 2003)).

1.7.4 Stem cells in the skin

Maintenance of the epidermal layer is achieved through a highly complex and orchestrated process. It is clear that epidermal SCs are closely associated with and appropriately activated through close communication with the cells comprising their SC niche. However, only limited knowledge exists about stem cell niches in the skin and the mechanisms that regulate epidermal stem cell behaviour. Lineage tracing suggests that the epidermal components are replenished from distinct SC compartments in the hair, IFE and sebaceous glands (Ghazizadeh *et al.* 2001), whereby it is not entirely clear whether the sebaceous gland contains its own SC population or whether it is maintained by hair SCs. It is generally accepted that a population of stem cells exists in the bulge region of the hair follicle and in clusters in the IFE. SCs from both, the hair and the IFE can show multipotential characteristics when exposed to the appropriate stimuli, and which differentiation pathway the SCs undergo seems to be dictated by the local microenvironment rather than by genetic pre-programming (Watt 1998, Panteleyev *et al.* 2001). For example, SCs in the hair follicle can be

induced to give rise to IFE and sebaceous cells (Taylor *et al.* 2000, Oshima *et al.* 2001) and *vice versa*, IFE SCs have been shown to differentiate into hair lineages and sebocytes (Reynolds *et al.* 1992, Ferraris *et al.* 1997). Similarly, sweat gland SCs can generate a stratified epithelium (Miller *et al.* 1998). This interconversion seems to be triggered upon exposure to the right microenvironmental cues, for example contact with the dermal papilla for hair follicle generation, or shifts in the balance of β-catenin (Silva-Vargas *et al.* 2005). An increase in β-catenin triggers hair follicle formation, whereas its deficiency triggers IFE formation (Huelsken *et al.* 2001). It appears that under normal conditions, SCs follow their normal pattern of differentiation, retaining multilineage potential for emergency situations, such as injury or stress.

Much research has focused on identifying markers of skin stem cells. As with other epithelial stem cells, some of the suggested stem cell markers include genes that confer SC characteristics such as self-renewal, quiescence, and maintaining cells in an undifferentiated state such as Wnt-inhibiting factor WIF1 and Dickkopf DKK3 (reviewed in (Watt *et al.* 2006)). Other putative molecular markers of epidermal SCs include CD34, which in humans is a well-described marker of haematopoietic SC, but is also expressed just below the bulge and in the ORS of anagen follicles (Poblet *et al.* 1994). Other potential stem cell markers are the bulge-specific keratin 15, as well as α6 and β1 integrins, and as recently suggested, CD200 (Terunuma *et al.* 2007).

1.7.4.1 Hair follicle stem cells

Controversy exists over the number of SC populations in the hair follicle. One suggested hair SC niche is located in the hair bulge, a specialised region in the ORS (Fuchs *et al.* 2001). Another SC pool has been suggested within the dermal papilla in the hair matrix at the base of the hair root, supposedly giving rise to the lineages of hair shaft and IRS (Kamimura *et al.* 1997). However, it is unclear whether these proposed stem cell niche locations actually represent separate SC pools, thus separating the

developmental origins of IRS and ORS, or whether one of them is a progenitor cell pool, derived from a common SC niche for ORS and IRS. Lineage tracing studies support the view of separate stem cell niches; matrix SCs responsible for generating the IRS lineages, bulge SCs generating the ORS (Blanpain *et al.* 2004, Legue *et al.* 2005). In contrast, the 'unitarian hypothesis' suggests that bulge SCs form the ORS and additionally move along the ORS to the matrix of the hair bulb, where they function as SCs to replenish IRS and hair shaft at the end of each hair growth cycle (Kopan *et al.* 2002).

1.7.4.2 The epidermal proliferative unit

Based on irradiation and kinetic studies, the existence of SCs in the IFE seems unquestionable. The exact stem cell number is still subject to debate. In the mouse epidermis, the proposed stem cell fraction ranges from 2% (Morris *et al.* 1985) and 4% (Bickenbach *et al.* 1998) to 10% (Withers 1967, Potten *et al.* 1988, Clausen *et al.* 1990, Kolodka *et al.* 1998, Mackenzie 1997) of the cells of the basal layer. The IFE SCs are believed to be organised in small clusters of basal quiescent cells, surrounded by proliferative transit amplifying cells (TAC), which after a few cell divisions undergo terminal differentiation (Jones *et al.* 1995, Jensen *et al.* 1999). It is not clear whether TACs are committed to differentiation into a certain cell lineage. They may be multipotential at the beginning of their division cycle or exist in gradients of commitment along the differentiation pathway.

In the mouse, it was shown that the *stratum spinosum* is organised into distinct cell columns of approximately 10 overlying cells, extending from the basal layer. It is believed that basally-located SC together with surrounding TACs form the so-called 'epidermal proliferative unit' (EPU) which gives rise to progenitors that divide and migrate upwards, thereby forming the column and providing a steady flow of differentiated keratinocytes to compensate for the constant shedding of cells from the epidermal surface (Mackenzie 1969, Christophers 1971, Potten 1974).

It is conceivable that humans too have a basal SC niche similar to the EPU, since the human and mouse epidermis are structurally organised in a similar fashion. However, cellular columns such as the ones observed in the mouse have not been identified to date (reviewed in (Potten 2002, Potten *et al.* 2004)). Nevertheless, expression of putative stem cell markers suggests that human epidermal stem cells reside in projections of the IFE into the dermis, known as rete ridges, above the mesenchymal dermal papilla. Keratin-19 (Michel *et al.* 1996) and β_1 integrin (Mackenzie 1970, Jones *et al.* 1995) have both been identified as potential markers of slow-cycling epidermal stem cells. Co-expression of both proteins has been observed in basally-located evenly spaced clusters within the rete ridges, making these locations likely candidates to home to the stem cell niche (Michel *et al.* 1996)

1.7.4.3 The bulge activation hypothesis

It has been argued that there is only one epidermal SC pool, located in the hair bulge, which gives rise to all epidermal cell lineages and all associated structures, and that what appears as IFE stem cells are in fact progenitor cells derived from this unique stem cell pool (Park *et al.* 2004). When cultured *in vitro*, bulge keratinocytes yield larger colonies than those from other skin sites. Other pieces of evidence show that the IFE stem cells are less numerous, less potent, display shorter longevity and are not capable of multipotential differentiation like bulge stem cells (Cotsarelis *et al.* 1990, Taylor *et al.* 2000, Alvarez-Dolado *et al.* 2003). A possible activation mechanism for this unique SC pool generating all structures of the human skin is explained by the 'bulge activation hypothesis'. Accordingly, cells in the dermal papilla of the hair bulb are capable of multipotential differentiation and generate both the hair shaft and the keratinocytes of the IFE. During catagen, the lower part of the hair follicle regresses, positioning the dermal papilla at the level of the bulge, where it remains throughout telogen. With the onset of anagen and the start of a new hair cycle, some cells from the bulge proliferate and move downward to form a new hair shaft, and other bulge-derived cells move upward to generate the

sebaceous gland and the keratinocytes of the IFE. Therefore, interactions between the mesenchymal cells from the dermal papilla and the epithelial cells of the hair bulge are responsible for stem cell activation. Several studies have confirmed that proliferation and differentiation of epidermal cells is dependent on direct contact between the bulge and the dermal papilla. 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).

1.7.5 Non-melanoma skin tumours

The skin is one of the most common sites of carcinogenesis, mostly due the constant exposure of the epidermal surface to environmental assaults, such as UV-exposure, chemicals and pollution. Amongst the wide variety of different types of skin cancer, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the most common.

It is hypothesised that tumours originate in the normal SCs of the tissue in question. As a logical conclusion one would expect that the different SC pools in the skin give rise to different typers of tumours. Indeed, epidermal tumours show variances in cell lineage differentiation, which may reflect the SC pool from which they originated. For example, SCCs have elements of IFE differentiation and hence are thought to originate from the IFE SC pool. Trichofolliculomas and pilomatrichomas show differentiation towards the hair shaft and are thus believed to originate from the SC pool that gives rise to these lineages.

Basal cell carcinomas (BCC) are the most common cutaneous malignancy in humans, accounting for up 75% of all skin cancers. Typically, BCCs appear on sun-exposed skin, are slow growing, and rarely metastasize. BCCs display a relatively homogeneous cellular appearance, resembling

keratinocytes. Although the exact etiology of BCC is unknown, a well-established relationship exists between BCC and the pilosebaceous unit, as tumours are most often discovered on hair-bearing areas. It has been suggested that they may originate in the ORS of the hair follicle, which is supported by the fact that sonic hedgehog (SHH) signalling, the malfunction of which results in BCC development, only occurs in hair follicles.

1.7.5.1 Clonality of skin tumours

The study of clonality has become a useful tool for studying cancer development by circumventing the technical difficulty of studying developmental dynamics *in vivo*. Mutation tracing studies have shown differences in clonality patterns between different types of skin tumours and revealed a clear link between actinic keratoses and SCCs, but attributed a different developmental origin to BCCs.

Despite a morphologically multifocal appearance, genetic analysis and three-dimensional reconstructions of BCCs have favoured a unicellular origin. This has been suggested by following non-random patterns of X chromosome inactivation, as well as by tracing the BCC-typical loss of heterozygosity (LOH) of chromosome 9 (9q22.3) (Walsh *et al.* 1996). These techniques showed that even anatomically-distinct BCCs may represent expansion of the same clone (Shulman *et al.* 2006). However, in some BCCs, the tracing of molecular markers showed genetic heterogeneity, which could indicate genuine polyclonality within the same tumour and reflect the independent origin of two or more tumours, or might show subclonality and represent the difference between following an early or late mutation in carcinogenesis (Asplund *et al.* 2005).

Controversy exists regarding the stromal origin of BCCs. Xenograft studies suggested that BCC generate their own 'specialised stroma', interspersed with cells derived from the host (Hales *et al.* 1989, Stamp *et al.* 1988), whereas other researchers believe that BCCs are embedded in a

connective tissue stroma at least in part of polyclonal origin (Asplund *et al.* 2005).

SCCs are believed to harbour more genetic heterogeneity. Comparison of the LOH patterns in oesophageal SCCs revealed that they are clonal neoplasms with considerable degree of divergence and genetic progression (Shima *et al.* 2006).

1.8 STEM CELL PLASTICITY

The capability of BM-derived cells to engraft into multiple organs is well documented and has in recent years attracted substantial attention from the research community. The plasticity of adult stem cells has challenged the widely-accepted belief that tissue stem cells are restricted to the production of adult lineages within their tissue of residence and simultaneously opened up new paths in the field of regenerative medicine, with the hope of finding treatments for a number of diseases such as cancer, cardiovascular disease, neurodegenerative disease and diabetes.

Transplanted adult BM cells have been shown to form adult cell types in many different tissues both, in mice and in humans (reviewed in (Poulsom *et al.* 2001)) (Figure 1.8, pg.74). In normal murine tissues, BM have been shown to engraft in the liver (Petersen *et al.* 1999, Lagasse *et al.* 2000), brain (Eglitis *et al.* 1997, Brazelton *et al.* 2000, Mezey *et al.* 2000), heart (Jackson *et al.* 2001, Orlic *et al.* 2001, Orlic 2002), kidney (Poulsom *et al.* 2001), intestine (Krause *et al.* 2001, Brittan *et al.* 2005) and skin (Krause *et al.* 2001, Badiavas *et al.* 2003, Kataoka *et al.* 2003, Harris *et al.* 2004, Brittan *et al.* 2005). Krause and colleagues confirmed the plasticity of a single purified BM cell by demonstrating donor-derived male cells eleven months post-transplant in the lung, the skin and throughout the gastrointestinal tract, including the oesophagus, stomach, small intestine and colon (Krause *et al.* 2001). In humans, plasticity of BM cells has been shown by engraftment into muscle (Ferrari *et al.* 1998), liver (Alison *et al.* 2000, Theise *et al.* 2000), vascular system of the skin and gut (Jiang *et al.*

2004), neurons (Cogle *et al.* 2004), Purkinje cells (Weimann *et al.* 2003), and the epithelium of the gastrointestinal tract (Spyridonidis *et al.* 2004). The extent of engraftment varies between different studies, tissues and detection techniques used. It ranges between 0.5-15% and is enhanced by injury. In general, it appears to reflect the turnover-rate of the tissue of destination.

BM cells can contribute to epithelial, mesenchymal, as well as vascular lineages. In the mouse lung, stomach, oesophagus, skin, kidney and adrenal glands they can contribute to large proportions of myofibroblasts and fibroblasts. Up to 65% of myofibroblasts in the mouse stomach were found to be BM-derived 6 weeks post-transplant (Direkze *et al.* 2003). Analysis of fibrotic livers from patients that had received a BM transplantation showed a significant BM-derived proportion of myofibroblasts and that the engraftment was not a transient, but a permanent event. Approximately 23% of myofibroblasts in one fibrotic liver were BM-derived 2 years post-transplant (Forbes *et al.* 2004). BM contains progenitor cells that can differentiate to form both endothelial cells and smooth muscle cells of blood vessels, particularly in areas of neo-vascularisation. BM-derived endothelial cells have been found in the human skin, stomach and small intestine up to 7 years post transplant. However, engraftment levels are low, ranging between 2.3% and 1.7% of endothelial cells in blood vessels of the skin and gut, respectively (Jiang *et al.* 2004). Plasticity is not restricted to BM cells, and even seems to underly bi-directional mechanisms, since BM cells have also been generated from muscle (Jackson *et al.* 1999) and neuronal cells (Bjornson *et al.* 1999), although the latter observation is disputed by some (Morshead *et al.* 2002).

The functionality of engrafted BM cells has been demonstrated in several pieces of research. Injection of BM cells from wt mice into transgenic mice with a deficiency in the liver enzyme fumarylaceto-acetate hydrolase as a model for the human metabolic liver disease Type 1 tyrosinaemia, resulted in normal liver enzyme expression conveyed by the engrafted cells into the mouse liver (Lagasse *et al.* 2000). Cu/Zn superoxide dismutase null

(SOD1) mice develop a lethal neurodegenerative disease similar to amyotrophic lateral sclerosis (ALS) in humans. When transplanted with wt BM, transdifferentiation of donor cells was shown into neurons, skeletal muscle fibres and cardiomyocytes, resulting in a delay in the onset of ALS and an increased lifespan (Corti *et al.* 2004). Another study has demonstrated a few BM-derived competent endocrine β-cells in the pancreatic islets of mice, inferring a potential cell-based therapy for diabetes mellitus (Ianus *et al.* 2003). In our laboratory, BM cells have been shown to alleviate symptoms of disease in a mouse model of chronic inflammatory bowel disease, suggesting the full functionality of normal BM-derived epithelial cells in the intestine (Bamba *et al.* 2006).

What triggers BM cells to transdifferentiate and home to the new microenvironment is unknown. Nevertheless, the fact that stem cell plasticity is also observed in adult tissues challenges the notion that stem cell pluripotentiality is a feature exclusive to embryonic stem cells. This presents a new outlook on regenerative medicine as adult stem cells, devoid of the ethical difficulties associated with the use of their embryonic counterparts.

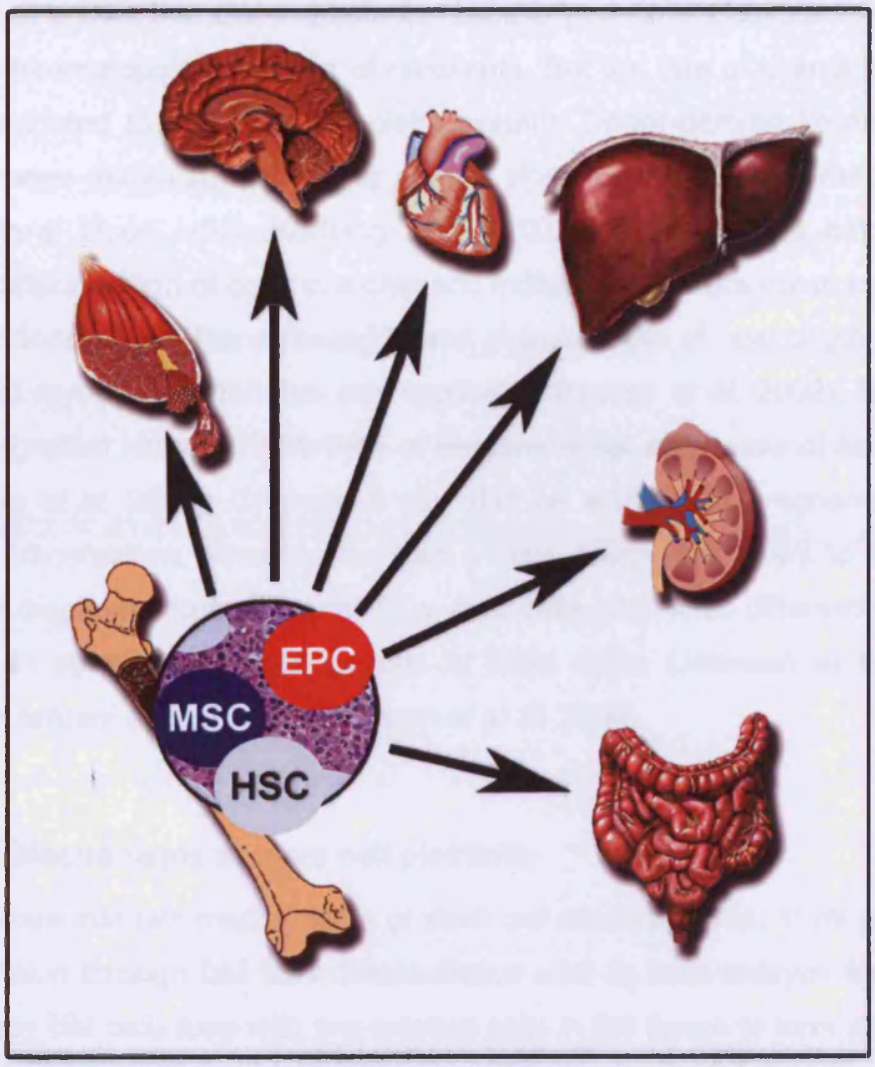

Figure 1.8. The plasticity of BM cells. BM cells have been shown to engraft into multiple organs, including the muscle, brain, heart, liver, kidney and gut.

1.8.1 Chimerism

It is well known that BM transplants result in permanent cellular chimerism in the haematopoietic system of recipients. But the fate of chimeric cells is not restricted to the haematopoietic system. Donor-derived keratinocytes have been demonstrated in the normal skin of female recipients of male peripheral blood HSC (Korbling *et al.* 2002). Moreover the pathway of transdifferentiation of cells in a chimeric individual appears not to be limited to the donor cells. Transplanted hearts showed cells of host origin in up to 10% of myocytes, arterioles and capillaries (Quaini *et al.* 2002). Similarly, within grafted kidneys, 23%-38% of mesenchymal cells were of host origin (Grimm *et al.* 2001). Chimerism can also be a result of pregnancies and blood transfusions. Women that had a male pregnancy years to decades earlier displayed mesenchymal SCs, liver cells and other differentiated cell types in several peripheral organs of male origin (Johnson *et al.* 2002, Khosrotehrani *et al.* 2004, O'Donoghue *et al.* 2004).

1.8.2 Mechanisms of stem cell plasticity

It appears that two mechanisms of stem cell plasticity exist: 1) *de novo* cell generation through BM transdifferentiation and; 2) heterokaryon formation, whereby BM cells fuse with pre-existing cells in the tissue to form a cell with multiple, genetically-variant nuclei. In cell fusion, it seems that the original stem cell markers become repressed and the transplanted cells transform the resident tissue cell they fuse with to a sustainable, functional cell. Heterokaryons appear to increase with tissue age (Weimann *et al.* 2003).

Co-cultures of embryonic stem (ES) cells with either adult neural stem cells (Jiang *et al.* 2002), or BM stem cells (Terada *et al.* 2002) displayed some fusion, albeit at very low frequency. Only 2-11 clones of 10^6 BM cells were heterokaryons (Terada *et al.* 2002). However, these studies involved genetically modified cells in culture and extracted from their niche environment, and may not reflect the mechanisms that apply *in vivo*.

Cell fusion has been proposed as the mechanism of BM cell incorporation in the generation of hepatocytes (Vassilopoulos *et al.* 2003, Wang *et al.* 2003, Willenbring *et al.* 2004). However, hepatocytes by nature are frequently polyploid, and it is thereby possible that the liver presents a unique environment for heterokaryon formation (reviewed in (Alison *et al.* 2004)). In the brains of mice and humans, transplanted BM stem cells have been shown to fuse with Purkinje cells (Weimann *et al.* 2003). Fusion has also been reported with skeletal (Camargo *et al.* 2003) and cardiac (Alvarez-Dolado *et al.* 2003, Nygren *et al.* 2004) muscle cells, and epithelial cells (Spees *et al.* 2003).

However, other reports could not confirm the occurrence of cell fusion in the human brain. Brain specimens from 3 female sex-mismatched BM transplant recipients showed that only one X chromosome was present in all male-derived neurons, indicating that transplanted BM cells are capable of neurogenesis, contributing to approximately 1% of all neurons for up to 6 years post-transplant (Cogle *et al.* 2004). In epithelia, fusion has not been reported in the gastrointestinal tract, based on a normal ratio of sex chromosomes (Korbling *et al.* 2002). Keratinocytes of BM origin are frequently observed in the mouse epidermis with no evidence of cell fusion (Borue *et al.* 2004).

1.8.3 Engraftment into the intestinal epithelium

Microinjection of 1-12 highly purified MSCs extracted from whole BM into 3.5-day-old mouse blastocysts resulted in chimeric mice with BM-derived epithelial cells in many somatic tissues, including the small intestine (Jiang *et al.* 2002). In humans, a combination of Y-FISH and cytokeratin immunohistochemistry, on specimens from female patients that had undergone sex-mismatched BM transplantation demonstrated epithelial cells of donor origin in the gastric cardia (Korbling *et al.* 2002). Similarly, donor-derived epithelial cells were detected in the oesophagus, stomach, small intestine and colon in four long-term BM transplant survivors who had developed GvHD or intestinal ulceration up to 8 years post-transplant,

demonstrating the long-term engraftment of transdifferentiated BM cells (Okamoto *et al.* 2002). Similarly, BM-derived cells have also been described in the colonic epithelium of 8 sex-mismatched BM recipients. Immunohistochemical staining for epithelial and lymphocyte markers combined with Y-FISH confirmed that the engrafted cells are not merely intraepithelial lymphocytes (Spyridonidis *et al.* 2004). Donor-derived cells occured as sporadic, randomly dispersed epithelial cells throughout the crypts and villi, and ranged from 0.04% (Spyridonidis *et al.* 2004) to approximately 10% (Jiang *et al.* 2002) of epithelial cells. These low levels of engraftment suggest that the BM-derived cells do not engraft as stem cells.

1.8.4 Engraftment into the epidermis

BM-derived cells have also been found in the mouse and human epidermis. In the normal murine skin, the engraftment of donor-derived cells ranges between 1-7% (Krause *et al.* 2001, Korbling *et al.* 2002, Badiavas *et al.* 2003, Kataoka *et al.* 2003, Harris *et al.* 2004, Brittan *et al.* 2005). The donor cells mostly occur as sporadically distributed single cells, which has lead to the suggestion that epithelial engraftment into the normal epithelium occurs merely as a form of cell mimicry by the donor-derived cells (Cogle *et al.* 2007). In the mouse epidermis, cytokeratin-positive donor-derived cells have also been demonstrated in postulated stem cell zones (Brittan *et al.* 2005), but the lack of observed clonal expansion suggested that they did not behave as stem cells (Hematti *et al.* 2002).

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). Kataoka and colleagues reported of a 'skin reconstitution assay', where adult BM cells were transplanted directly onto the wounded mouse epidermis. Within three weeks, epidermal keratinocytes, follicular epithelial cells, sebaceous gland cells, dendritic cells and endothelial cells of BM origin were observed (Kataoka *et al.* 2003). In humans, little research has been carried out to determine the engraftment of BM-derived cells in the normal skin. A recent study could

not confirm any engraftment of BM cells into the normal skin of humans, but only in patients with graft-versus-host disease, particularly in areas of heightened tissue damage (Murata *et al.* 2007). In another study, researchers were able to detect BM-derived epidermal cells in human female recipients of male HSC transplants as late as 354 days post-transplantation (Korbling *et al.* 2002).

1.8.5 BM engraftment in stem cell niches

Research in our lab has demonstrated that BM-derived cells can home to known stem cell niches, such as the CD34-positive bulge region of the hair follicle and the epidermal proliferative unit in the interfollicular epidermis (Brittan *et al.* 2005), and thus can presumably contribute to epithelial homeostasis. Cells that are engrafted and act as stem cells then have a sufficient lifespan to acquire the mutations required for neoplastic transformation and in this manner may give rise to donor-derived tumours. This notion is challenged by a study carried out by Hematti *et al.*, who could not detect cells capable of growing under conditions favourable for keratinocyte stem cells after haematopoietic stem cell transplantation (Hematti *et al.* 2002).

1.9 BONE MARROW CONTRIBUTION TO INJURY AND TUMOURS

The engraftment of BM cells is markedly increased by injury such as epidermal wounding or inflammation. In the mouse epidermis for example, wounding increased the extent of BM cell engraftment to 13% of epithelial cells. In a mouse model of experimental colitis, BM cells showed increased engraftment in the inflamed intestinal epithelium, and they contributed to the repair of the injured tissue (Brittan *et al.* 2005). Similarly increased BM cell engraftment was observed in murine models of cerebral infarction (Hess *et al.* 2002), and myocardial infarction (Jackson *et al.* 2001, Orlic 2001, Orlic *et al.* 2002). In humans, BM contribution to intestinal epithelium showed a 5–50-fold elevation in tissue damaged by GvHD or intestinal

ulceration, compared to intact epithelium (Okamoto *et al.* 2002, Spyridonidis *et al.* 2004). It is proposed that BM stem cells respond to specific signals from damaged tissues, causing them to migrate to, engraft in and differentiate into cells within the tissue, thereby contributing to regeneration.

In disease scenarios, BM engraftment appears to underlie different principles than in normal tissues. In areas of chronic inflammation, engraftment is not only profoundly enhanced in quantity, but donor-derived cells are seen in patterns that are typical for cells derived from a common ancestor (Houghton *et al.* 2004). It appears that in most cases, an environment of continuous damage, such as chronic inflammation, is required for BM-derived cells to engraft and act as stem cells in epithelia. A possible explanation for this scenario is that permanent tissue damage eventually results in failure of the resident tissue stem cells, whereupon BM cells are recruited to fill the free stem cell niche. Acting as the new tissue stem cells, the BM-derived cells have the longevity to acquire the changes required to transform into cancer stem cells that then may give rise to entire donor-derived, post-transplant tumours (Figure 1.9, pg.80). In accordance with this theory, Houghton *et al.* showed donor-derived gastric cancer in a mouse model of chronic inflammation and progression to gastric cancer (Houghton *et al.* 2004).

BM cells can also contribute to the tumour stroma and to neovascularisation of tumours (Duda *et al.* 2006, Nolan *et al.* 2007). In our laboratory, it has been demonstrated in a mouse model of pancreatic insulinoma that transplanted BM cells can contribute to the supporting stromal myofibroblasts (Direkze *et al.* 2004).

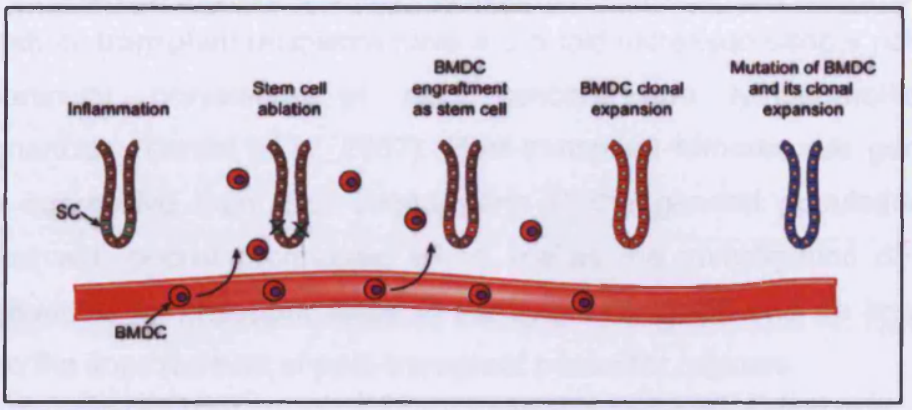

Figure 1.9. A new paradigm of epithelial tumour development using the small intestine as an example. Continued tissue damage leads to loss of the indigenous stem cell compartment and its replacement by cells from the BM. As acting stem cells, the engrafted BM cells have now sufficient lifespan to undergo neoplastic transformation and give rise to entire BM-derived cancers. (BM-derived cells are red, mutated BM-derived cells are blue). (Figure taken from Burkert *et al.* 2006).

1.10 DONOR-DERIVED CELLS IN POST-TRANSPLANT CANCERS

The development of malignancies is a well-known side effect of tissue and cell transplantations, attributable to immunosuppression, UV-irradiation and human papillomavirus (HPV) infection. Compared to the general population, transplant recipients have a 3-5-fold increased cancer risk, with a particular prevalence of skin cancers and lymphoproliferative malignancies (Dantal *et al.* 2007). Post-transplant tumours are generally more aggressive than their counterparts in the general population and present with poorer prognoses, which makes the investigation of these malignancies an important issue in transplant medicine and an important step in the improvement of post-transplant treatment regimes.

Cells of donor-origin have been observed in post-transplant tumours to a variable extent. In most cases, the engraftment of BM cells in tumours appears to occur in a sporadic fashion, similar to the engraftment of transplanted cells in normal tissues. However, it has also been shown that post-transplant lesions can be completely composed of BM-derived cells. Papadopoulos and colleagues (1994) reported on a BM transplant patient who developed leukaemia post-transplant with the entire leukaemic clone derived from donor-derived cells. A pioneering study by Houghton *et al.* (2004) proved the BM origin of entire solid tumours in a mouse model of gastric cancer. Since then, adherent cells from whole BM have been shown to be capable of transformation *in vitro* into malignant cells expressing multiple tissue markers when treated with 3-methylcholanthrene, and when the transformed cells were injected subcutaneously into nude mice, they were capable of generating tumours with multiple phenotypes including epithelial, neural, muscular tumours, tumours of fibroblasts and tumours of endothelial origin (Liu *et al.* 2006).

In humans, engraftment of donor-derived cells after solid organ transplantations has also been reported. In kidney transplant recipients, leukaemic clones completely derived from the donor have been found (Bodo *et al.* 1999). Moreover, cells stemming from the transplanted organ were shown in Kaposi sarcomas in recipients of liver allografts (Barozzi *et al.* 2003). Notably, these are tumours of a haematopoietic origin that could be derived from haematopoietic progenitors carried over with the transplanted organ. However, Aractingi and colleagues described one basal cell carcinoma (BCC), which appeared to be composed mostly of male cells indicating that transplanted cells may be able to undergo neoplastic transformation in the host. However, a possible origin of this tumour in haematopoietic cells from an earlier blood transfusion could not be excluded.

Donor-derived cells could be haematopoietic stem cells carried over with the transplanted organ, or a population of cells residing within the organ, which is capable of plasticity. Whatever the case, these results demonstrate that neoplastic progenitors can be transmitted with the transplanted organ, or that cells from the donated organ are capable to transform into cancer-generating cells in the host.

1.11 THE CONNECTION BETWEEN MICROENVIRONMENT AND ENGRAFTED CELLS

Our knowledge of the stem cell niche in the skin indicates that the microenvironment may be an extremely important factor in cellular development. The transdifferentiation and incorporation of transplanted BM cells as functional members of their new environment also highlights the effect of local cellular and biochemical cues on cell behaviour. However, it is not clear to what extent cellular behaviour and environment is governed by the cells' intrinsic genetic programming, or by their immediate microenvironment, respectively. More insight into this matter would yield important information for the field of transplant medicine. It may be possible

that transplanted cells with an adverse genetic predisposition, upon engraftment into their new tissue of destination, act according to their genetic program and for example, transform to cancer stem cells, generating a tumour in the new host. Similarly, do transplanted cells incorporated into a diseased microenvironment merely develop as part of the tissue and adopt the disease phenotype or can they exert an influence according to their genetic predisposition?

Evidence for at least a certain degree of impact exerted by genetic programming is provided by the proven functionality of engrafted BM cells and the reversal or delay of disease phenotypes in mouse models of liver disease Type 1 tyrosinaemia (Lagasse *et al.* 2000), neurodegenerative disease amyotrophic lateral sclerosis (ALS) (Corti *et al.* 2004), and diabetes (Ianus *et al.* 2003). In a mouse model of colitis, transplanted BM-derived cells were able to alleviate the symptoms and contribute to epithelial healing (Bamba *et al.* 2006). Recently, a study has shown that in a rat model of MI associated with hypertension, administration of BM cells significantly improved cardiac function (de Macedo Braga *et al.* 2007). This evidence indicated that transplanted cells are able to affect their immediate surroundings.

On the other hand, the impact of the immediate cellular environment is highlighted in the event of chronic inflammation, where it has been shown that intercellular factors such as adhesion molecules or cytokines are ultimately responsible for influencing cells to transform. A murine model of gastric cancer, where BM-derived cells engrafted into the gastric mucosa upon exposure to *H.Pylori* infection eventually transform to give rise to gastric cancer, is a prime example for the effect of the surroundings on newly-incorporated cells (Houghton *et al.* 2004).

Together, this suggests that the effects between incorporated cells and their local microenvironment are reciprocal, and similarly challenges the notion of incorporated cells merely contributing to their destination tissue as by cell mimicry as suggested by Cogle and colleagues (Cogle *et al.* 2007).

If the genetic predisposition of a transplanted cell has an impact on the host, this implies a new risk factor for transplant patients. On the other hand, an immediate influence of incorporated cells might also present new exciting therapeutic potential.

AIMS AND OBJECTIVES

The search for markers of stem cells and cancer stem cells is a prominent objective of the research community. In this project, we aimed to test the Side Population phenotype, as well as surface markers CD44 and CD133 for their applicability as stem cell markers in colorectal cancer cell lines and primary colorectal adenocarcinoma cells, respectively. Studies involving cells lines were outlined as a comprehensive assessment of phenotypic and behavioural stem cell-typical characteristics of FAC-sorted SP and non-SP cells, examining the expression of other putative stem cell markers, clonogenicity, multipotentiality *in vitro* and *in vivo*, tumorigenic ability *in vivo*, drug effluxing capacity and proliferation efficacy of both populations. Next, we seeked to test the differential expression characteristics of CD133 and CD44 and their combination on colonic normal tissue and adenocarcinomas, and their ability to act as stem cells by a sphere-assay *in vitro* and upon injection in nude mice. A stem cell enriched population would be expected to result in formation of normal crypts and xenograft tumours.

The next main objective of this thesis was to look into the origin of epithelial tumours in a transplant setting. Following several recent reports of donor-derived post-transplant tumours, we set out to perform the first large-scale study investigating this phenomenon in order to determine whether the seeding of cancer-originating cells from the host is a realistic and, so far, unknown risk for transplant patients. For this study, skin tumours from 176 female recipients of male renal allografts were selected for molecular procedures such as qPCR to quantify the amount of male DNA within the tumours, as well as immunohistochemical techniques like X and XY-FISH and combined Y-FISH and immunohistochemistry for various cell type - specific antigens to locate the Y-positive cells and identify their cell type.

To investigate the possibility that the genetic predisposition of transplanted cells could have a detrimental effect on the host and the reciprocal effects of transplanted and immediate host cellular environment in more detail and in an experimental setting, we decided to employ a mouse model of spontaneous skin carcinogenesis. Reciprocal injections of BM into lethally irradiated mice and follow-up assessment of tumour formation, tumour latency and the incorporation of donor-derived cells into the formed skin tumours using the techniques described above was to elucidate the phenotypic effects of donor and host cells on each other.

Finally, we aimed to gain more understanding about skin tumour development and lineage relationships in the skin. The method of choice for this study was tracing a clonality marker; the phenotypic manifestation of mitochondrial mutations in the genes coding for cytochrome c oxidase, the deficiency of which results in a blue staining upon an enzyme histochemical assay. Mutation analysis and sequencing the mitochondrial genome of microdissected cells from cytoochrome oxidase deficient and competent areas can then reveal molecular relationships between skin tumours and the normal tissue, between different cell lineages and potentially reveal information regarding field cancerisation in the human skin.

CHAPTER II

MATERIALS AND METHODS

2.1 OUTLINES OF INDIVIDUAL EXPERIMENTAL STUDIES

2.1.1 Side Populations of human gastrointestinal cancer cell lines are not enriched in stem cells

Following observations from several tissues, cancers, and cell lines, it was suggested that the SP phenotype may represent a universal stem cell marker. We tested whether this hypothesis holds true in cell lines derived from various gastrointestinal cancer cell lines.

2.1.1.1 Phenotypic characteristics

SP and non-SP cells were tested for expression of putative stem cell surface markers by multicolour FAC analysis using antibodies directly labelled with a fluorescent protein. Expression of CD34, CD44, CD117, CD133 and BCRP1 was tested *in vitro* before and after culturing of SP and non-SP fractions before and after xenograft formation *in vivo*. Additionally, we examined expression of putative stem cell markers on colonies generated from single cell-sorted SP and non-SP cells by immmunohistochemistry, including β-catenin, HES-1, OCT-3/4, MSI-1, MDR-1, CD34, CD117 and CD133.

2.1.1.2 Behavioural characteristics

Stem-cell typical behaviour, such as self-renewal and multilineage differentiation potential of SP and non-SP fractions was experimentally tested by clonogenic assays, sodium butyrate-triggered differentiation assays *in vitro*, as well as by injection of sorted cells into nude mice at limiting dilution for xenograft formation. Immunohistochemistry on xenograft tumours was performed to reveal cell type composition of xenograft tumours.

2.1.2 Characterisation of cells expressing the putative stem cell markers CD44 and CD133 from human adenocarcinoma and normal epithelium of the colon

The surface proteins CD44 and CD133 have both been suggested to identify cell populations that are enriched in stem cells. We examined, whether the combination of both markers results in a further purified stem cell population. Stem cell characteristics were experimentally tested by a sphere-formation assay *in vitro* and xenograft formation *in vivo*. Freshly resected specimens of colonic adenocarcinomas and adjacent normal epithelium were processed into single cell suspensions for multicolour FAC sorting. Isolated were the CD45-negative populations that expressed either CD44 or CD133 or both markers together, as well as their negative counterparts. The sorted cell fractions were incubated in serum-free medium to foster sphere formation, as well as injected s.c. into nude mice in a mixture of PBS:matrigel (1:2) for xenograft formation. Additionally, double fluorescent immunohistochemistry was performed for both surface markers on paraffin-embedded tissue sections to visualise the locations of double-positive cells.

2.1.3 Donor-derived skin tumours after kidney transplantation

It is well known that transplanted BM cells can show considerable plasticity, including their incorporation into cancers. However, little is known about the contribution of cells derived from solid organ transplants to post-transplant cancers. 176 skin cancers from 35 sex-mismatched female kidney transplant recipients were examined for the presence of Y-chromsomal DNA by qPCR. Positive samples were further analysed by XY-FISH to reveal location and distribution of the donor-derived cells. Preferential cell-type specific engraftment of Y-positive cells was tested by a technique combining Y-FISH and immunohistochemistry for the cell type-specific antigens α-SMA (stromal myofibroblasts), pan-cytokeratin (epithelial cells), CD34 (endothelial progenitors, potential stem cells), and CD45 (leukocytes). The same technique was used to elucidate whether the donor-derived cells are prone to HPV infection.

2.1.4 Effect of local microenvironment and genetic predisposition on cancer development in a mouse model of skin carcinogenesis

In this study, we evaluated the impact of genetic pre-disposition of transplanted cells in the host organism, and *vice versa*, the impact that the local microenvironment exhibits on the development of transplanted cells. Reciprocal BM transplants were carried out with lethally irradiated hosts and an injection dose of 2 million whole BM cells, involving C57/Black 6 wild type (wt) mice and transgenic HPV8 mice that develop spontaneous skin tumours. Control transplant groups were included. Resulting skin tumours were examined by Y-FISH and immunohistochemistry for the presence of donor-derived cells, the extent of engraftment, and cell type-specificity of donor cell-engraftment.

2.1.5 Clonality in skin tumours and implications for lineage relationships in the skin and the cell origins of BCCs

To test lineage relationships and genetic origins of various types of skin tumours, a well-established method was employed, which is used in our laboratory to study clonality in the gastrointestinal system. We tested frozen sections from skin cancers of squamous and basal origin for cytochrome c oxidase activity by enzyme histochemistry and immunohistochemistry. Cells were extracted from cytochrome c oxidase-deficient and competent areas by laser-assisted microdissection, and their mt genome was sequenced following a nested PCR method. Individual mutations were directly compared to evaluate clonality and trace developmental origins within skin tumours.

2.2 ANIMAL STUDIES

All animal procedures were carried out under UK Home Office procedural regulations after approval of an ethical committee. All mice were maintained in the Biological Resource Unit, Cancer Research UK, London Research Institute.

2.2.1 Xenograft experiments with cell populations from human gastrointestinal cell lines

Xenograft experiments were performed using 4 selected cell lines (HT29, HGT101, Caco2, HRA19a1.1). FAC-sorted SP or non-SP population cells in 200 μl medium/matrigel (1:1) were injected s.c. into 4-8 week-old nude mice at limiting dilution (Table 2.1, pg.107). Before injection, sorted cells were stored on ice for no more than 1 hour. Tumour development was monitored starting from the second week, and then measured weekly. All animals were culled by CO_2 inhalation and cervical dislocation 16 weeks after injection or when tumour size passed the limit of 15 mm in diameter. Harvested tumours were cut in half. One half of each tumour was fixed in 4% neutral buffered formalin (NBF) and embedded in paraffin for H&E

staining and immunohistochemistry; the other half was minced, digested in a mixture of 3% collagenase/5% dispase at 37°C for 1 hr, passed through 70-micron cell strainers and incubated in the culture conditions previously described, to establish cell cultures for future experiments.

2.2.2 Xenograft experiments with primary human cells

FAC-sorted cells from freshly resected adenocarcinomas and normal colonic epithelium were injected s.c. into nude mice in 200 μl PBS/matrigel (1:2) in cell doses of 100 and 1000 cells per injection (Table 2.2, pg.108). Before injection, sorted cells were stored on ice for no more than 2 hours and viability was tested immediately before injection on a Vi-Celll XR™ cell viability analyser (Beckman Coulter). Cells and control cells were injected s.c. ventrally into opposite flanks. Mice were earmarked individually. Tumour development was monitored weekly. All mice were culled 20 weeks after injection as described above. Subcutaneous tissue was dissected where accumulation of matrigel marked the site of injection, fixed in 4% NBF, and embedded in paraffin for H&E staining and mouse-human pan-centromeric *in situ* hybridisation.

2.2.3 Bone marrow transplants

2.2.3.1 Mouse strains

The mice strains used for BM transplantations were either C57/Black 6 wild type (wt) mice or transgenic mice with genomic incorporation of the complete early region with its early oncoproteins E2, E6, and E7 of the cutaneous Human papillomavirus 8 (HPV8) on a FVB/N background. The HPV8 transgenes are integrated downstream of the cytokeratin-14 promoter, which is known to target expression to the epidermis and developing hair follicles of mouse embryos, as well as the basal and spinous layers of mouse adult skin. Transgenic heterozygote mice develop single or multifocal epidermal lesions with a median age of tumour development of 8.4 weeks. Benign tumours identified by papillomatosis,

hyperkeratosis, and acanthosis develop in 91% of transgenic mice, 25% of which proceed to moderate or severe epidermal dysplasia. SCCs develop in 6% of these mice (Schaper *et al.* 2005).

2.2.3.2 Transplants

Six week old female recipient mice had their BM ablated by 12 Gray whole body irradiation in a divided dose, 4 hours apart, using an IBL 637 gamma irradiator equipped with a caesium 137 source (Cis-Bio International). This was immediately followed by tail vein injection of approximately 1 x 10^6 whole BM cells from 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 200x*g* for 2 x 5 minutes, the supernatant was discarded, and cells resuspended in 0.2 ml sterile PBS. The number of cells was calculated using a haemocytometer. Cells were stored on ice for no more than 2 hours before transplant. Following transplants, the mice were housed in sterile conditions. All mice were killed by CO_2-inhalation and cervical dislocation 20 weeks post-transplant or when tumour burden was unbearable (exceeding the threshold diameter of 20 mm or multiple tumour foci). Epidermal lesions, as well as samples of unwounded and wounded skin were fixed in NBF for 24 hours at room temperature, transferred to 70% ethanol and embedded in paraffin.

2.2.4 Cutaneous wounds

Four weeks post-transplantation, two full-thickness circular wounds, each 3 mm in diameter, were made to the dorsal skin of the mice in the groups indicated in Table 2.3 (pg.109). Mice were killed 20 weeks post-transplant or when tumour burden was unbearable (exceeding the threshold diameter of 20 mm or multiple tumour foci).

2.3 HUMAN STUDIES

2.3.1 Kidney transplant patients

42 female recipients of male kidney allografts that had developed skin lesions following the transplant were identified. Archival paraffin-embedded biopsies from 176 skin lesions with varying histopathological diagnoses were used for this study (Table 2.4, pg.110). Ethical approval was sought and obtained as per the requirements of the United Kingdom Human Tissue Act (2006).

2.3.2 Skin cancer patients

For the study described in chapter VII, patients that underwent surgical resection of skin tumours were selected. Specimens were snap-frozen in liquid nitrogen-cooled isopentane or fixed in 4% PFA and embedded in paraffin. Ethical approval was sought and obtained as per the requirements of the United Kingdom Human Tissue Act (2006).

2.4 TISSUE CULTURE

2.4.1 Cell Culture

2.4.1.1 Cell lines and maintenance

The human gastrointestinal cancer cell lines used for our experiments were of colorectal (HT29, HRA19a1.1 [doubly cloned from HRA19 (Kirkland *et al.* 1994)], Col1, Col29 [sublines of HCA7 (Marsh *et al.* 1993)], SW480, SW1222) and gastric (HGT101 (Laboisse *et al.* 1982)) origin. HRA19a1.1 cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, PAA) supplemented with 2mM glutamine, 0.06 g/L penicillin, 0.1 g/L streptomycin and 10% Foetal Bovine Serum (FBS) (Sera Laboratories International). The remaining cell lines were maintained in DMEM, supplemented with penicillin, streptomycin, and 10% FBS. All cell lines were incubated at 37°C in humidified atmosphere containing 8% CO_2.

2.4.1.2 Clonogenic assays

To analyse clonogenic and morphological properties of SP and non-SP cells of HGT101, HT29 and Caco2 cell lines, single cells of both fractions were sorted into 96-well plates and cultured as described above. The medium was changed every 2-3 days. After 3 weeks, cells were fixed in 4% PFA in PBS for 15 min at room temperature. Cloning efficiency was measured to indicate the percentage of cells that initiated a clone. Size and morphology of the clones was assessed, whereby the different morphological behaviour displayed by Caco2 clones was taken into account by a classification system separate from HT29 and HGT101 cells. HGT101 and HT29 colonies were grouped into 4 different categories as: (i) very small (<50 cells), (ii) Holoclones: tight colonies displaying a clearly defined border, (iii) Paraclones: dispersed colony morphology (iv) Meroclones: intermediate colonies - containing areas of tightly-packed as well as dispersed cells with no clear border. Caco2 colonies were categorised by size as small (<50 cells), intermediate (spanning up to half of the field of view on a phase-contrast microscope with an overall magnification of x40, and large (spanning more than half of the field of view). Experiments were performed in triplicate, and repeated twice.

2.4.1.3 Re-analysis of cultured cell fractions

To examine long-term behaviour of sorted SP and non-SP cells, cells were seeded at clonal density in T25 culture flasks (Becton Dickinson) (1500 cells/flask) and maintained in the culture conditions described above. Media were changed every 2-3 days. After 3 weeks, cells were prepared for flow-cytometric analyses as described above.

2.4.1.4 Differentiation assays

Purified SP and non-SP cells were plated at $1x10^3$ cells/chamber in plastic 8-chamber slides (LabTek™) and maintained as described above. When cells reached confluency, medium supplemented with 2 mM sodium butyrate (NaB) was added to trigger differentiation (Stokrova *et al.* 1999, Cai *et al.* 2004, Hossain *et al.* 2005) and after 72 hrs, cells were fixed in 4% PFA in PBS.

2.4.2 Fresh tissue

Freshly resected human colonic adenocarcinoma and normal colon samples were washed 5x thoroughly in PBS supplemented with 1 mM dithiolthreitol (DTT) (Invitrogen) to remove mucous. Normal colon was then incubated in E4 medium lacking Mg2+ and Ca2+, supplemented with 30mM EDTA at 37°C for 15 min to dislodge crypts, followed by vigorous shaking by hand. The supernatant was transferred to a Falcon flask and spun at 200*g* for 5 min. The pellet was re-suspended in 5 ml 0.2% collagenase/0.5% dispase solution in PBS and incubated in a shaking waterbath at 37°C for 1 hr. Tumour and xenograft samples were covered with 0.2% collagenase/0.5% dispase and minced with two scalpels, followed by incubation in a shaking waterbath at 37°C for 2-3 hrs. Both, tumour and normal digested materials were passed through 70μ nylon meshs and spun at 200*g* for 5 min. The pellets were re-suspended in ammonium chloride and left on ice for 10 min to lyse red blood cells. The cells were spun at 200*g* for 5 min, re-suspended in PBS and counted using a haemocytometer. The cells were spun again and re-suspended at appropriate dilutions in PBS. If cells were to be used for the sphere formation assay *in vitro* (chapter IV), they were re-suspended in DMEM without the addition of FCS.

2.5 FLUORESCENCE-ACTIVATED CELL ANALYSIS AND SORTING

2.5.1 Side population analysis

To identify the presence of SPs, the protocol developed by Goodell *et al.*, (Goodell *et al.* 1997) was largely followed. In brief, near-confluent cells were trypsinised, washed with phosphate-buffered saline (PBS), resuspended in DMEM/3% FCS at $1x10^6$ cells/ml and passed through 40-micron cell strainers (BD Falcon) to achieve single-cell suspensions. To verify the SP phenotype, we used the ABC-transporter blocking ability of reserpine (100 μmol/L) as described by Zhou *et al.* (Zhou *et al.* 2001). Optimal staining conditions, defined as the Hoechst concentration and incubation time where the SP phenotype could be maximally inhibited by reserpine, were determined in time-course experiments with Hoechst concentrations ranging between 5-10 μg/ml and incubation times of 30-90 min (Table 2.5, pg.111). Cells were then transferred to ice and labelled with 1 μg/ml pròpidium iodide (PI) to discriminate dead cells. The stained cells were analysed using a LSR II Flow cytometer (BD Biosciences). The Hoechst dye was excited by UV laser at 355 nm and its fluorescence measured with 440/40 nm (Hoechst blue) and 620 LP (long-pass, Hoechst red) optical filters.

Three cell lines (HT29, Caco2, HGT101) were selected for further experiments based on their origin and growth characteristics. *In vivo* experiments were carried out with 4 cell lines, using HRA19a1.1 cells in addition to the three above mentioned cell lines.

2.5.2 Fluorescence-activated cell sorting

For sorting of SPs, non-SPs and whole Populations, cells were stained according to the optimal conditions determined above for each of the selected cell lines. Cells were sorted using a MoFlo™ High Performance Cell Sorter (Dakocytomation). The Hoechst dye was excited at 350-356.4 nm and fluorescence was measured at 424/44 nm (Hoechst blue) and 620 nm (Hoechst red). Doublets and PI-stained dead cells were electronically gated out. Inhibition of the SP phenotype by reserpine was used as guidance for drawing of appropriate sorting gates.

2.5.3 Multicolour immunofluorescence flow cytometric analysis

2.5.3.1 Staining of live cells for putative stem cell surface markers

To determine whether SP cells differ from non-SP cells in surface marker expression; we analysed the selected cell lines (HGT101, HT29 and Caco2) for expression of the putative stem cell surface markers CD34, CD44, CD117 (c-Kit), CD133 (prominin1) and BCRP1 (ABCG2). Cells were first stained with Hoechst 33342 under previously determined optimal conditions. Excess Hoechst dye was removed by pelleting cells at 4°C and re-suspending them at $1x10^7$ cells/ml in DMEM/10% FBS. One million cells were incubated on ice for 20 min with fluorescently conjugated antibodies, details of which are outlined in Table 2.5 (pg.111). In control experiments, cells were also stained with each antibody individually, and all antibodies less one to draw and confirm correct gates marking antibody negativity at analysis. All antibodies were used undiluted. The stained cells were analysed using the LSR II Flow cytometer. Fluorescence signals of the conjugated antibodies were measured at wavelengths outlined in Table 2.6 (pg.112). Data were analyzed using FloJo computer software (Treestar).

2.5.2.2 Staining of fixed cells for Ki-67

Cells were fixed by adding in ice cold 70% ethanol dropwise to the cell pellet and stored overnight at -20°C. Two washes were performed in 20ml staining buffer (PBS with 1%FCS, 0.09% NaN_3) with interim centrifugation at 200x*g* for 5min, whereupon cells were resuspended to $1x10^7$ cells/ml. A FITC-labelled anti-Ki-67 antibody (20µl at appropriate dilution) was added to 100µl of the cell suspension and incubated at RT in the dark for 1hr. The cells were then washed twice in 5ml staining buffer, again with centrifugations at 200x*g* for 5min. Finally, the stained cells were resuspended in 500µl staining buffer. After addition of 3 drops of propidium iodide (PI) solution, flow cytometric analysis was performed.

2.6 IMMUNOHISTOCHEMISTRY AND *IN SITU* HYBRIDISATION

Immunohistochemistry for various antigens was used to establish cell phenotype. In tissues of female mice that received a BM transplant from a male mouse donor and sex-mismatched female transplant recipients, *in situ* hybridisation for the XY chromosomes or the Y chromosome was used to distinguish the transplanted cells. The combination of immunohistochemistry and *in situ* hybridisation permitted both tracing of cell origin and phenotypical analyses.

Information regarding primary and secondary antibodies is presented in Tables 2.7 and 2.8. For all immunohistochemical and *in situ* hybridisation protocols, appropriate positive and negative controls were used. Prior to routine use of any newly acquired antibody, the optimal dilution and conditions were determined by testing serial dilutions and various antigen retrieval techniques on positive control sections of fixed tissue. Normal sera and secondary antibodies were used at the highest concentration (i.e., lowest dilution factor) recommended by the manufacturer.

2.6.1 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 4 μm or 10μm using a Leica RM2235 microtome, depending on whether sections were used for immunohistochemistry or laser capture microdissection, respectively. Tissue sections were placed on frosted glass microscope slides (Thermo Shandon COLORFROST®) and dried overnight at 37°C. Sections were stored at room temperature in sealed slide boxes.

2.6.2 Dewaxing and blocking of endogenous peroxidases

Tissue sections were dewaxed by immersion in sulphur-free xylene (VWR) for 5 min, followed by rehydration in decreasing concentrations of ethanol (100%, 95%, 80%, 70% and 50%) for 3 min each. Sections were then transferred to distilled water. Where immunohistochemistry was performed using the horseradish-peroxidase (HRP) detection system, endogenous peroxidases were blocked by treating sections with 30% hydrogen peroxide (VWR) in methanol, rinsed in tap water, and washed in PBS.

2.6.3 Immunohistochemistry protocol

Antigen retrieval was performed either by microwave treatment in boiling sodium citrate buffer, pH 6.0 at 700 W for the time indicated in Table 2.8 (pg.114), by incubation in bovine trypsin (VWR) for 15 minutes at 37°C, or by pressure cooking in boiling sodium citrate buffer, pH 6.0. Sections were then rinsed in tap water, washed in PBS, and pre-incubated in normal serum (DAKO) from the species the secondary antibody was raised in for 15 minutes, diluted at 1:25 in PBS. Sections were then incubated in the appropriate dilution of primary antibody, followed by incubation in a species-specific secondary antibody. All secondary antibodies were biotinylated, except in the case of anti-CD44 and anti-CD133 staining of human adenocarcinoma and normal colonic tissue (Chapter IV), where directly labelled fluorescent secondary antibodies were used. A tertiary layer of either streptavidin-horseradish peroxidase (strep-HRP)(DAKO)

diluted to 1:500, or streptavidin-alkaline phosphatase (strep-AP)(DAKO) diluted to 1:50, was finally applied. Primary, secondary and tertiary layers were all diluted in PBS and incubations were carried out for 35 minutes at room temperature unless otherwise indicated in Table 2.8 (pg.114). 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 3,3'-diaminobenzidine (DAB) (Sigma), and Strep-AP was detected using the Vector Red Alkaline Phosphatase Substrate Kit 1 (Vector Laboratories) for 2-15 min until the brown colour of the DAB or the red colour of the Vector Red kit could be detected macroscopically. Sections were then washed in PBS for 2 x 5 minutes, rinsed in tap water and counterstained with haematoxylin and eosin, unless immunohistochemistry was followed by *in situ* hybridisation, where the sections were immediately subjected to the first step of the procedure.

2.6.3.1 Double fluorescent immunohistochemistry

For double immunohistochemical detection of CD44 and CD133, sections were dewaxed and subjected to antigen retrieval by pressure-cooking in boiling sodium citrate buffer, pH 6.0, for 3 min. This was followed by incubation in goat serum (DAKO) for 25 min at a dilution of 1:25 in PBS. Anti-CD44 was applied (1:100) for 40 min at room temperature, followed by a FITC-labelled anti-mouse IgG secondary antibody (1:200) (Invitrogen). Subsequently, anti-CD133 (1:500) was applied for an overnight incubation at 4°C, followed by a secondary Cy-3 labelled anti-rabbit IgG (1:1000)(Invitrogen) at room temperature for 35 min. Sections were then mounted in VECTASHIELD® Hard Set™ mounting medium containing DAPI (Vector Laboratories) for nuclear visualisation. Between each step of the protocol, sections were washed twice in PBS for 5 min.

2.6.4 *In situ* hybridisation

After dewaxing, sections were incubated in 1 M sodium thiocyanate (Sigma) in distilled water for 10 minutes at 80° C. The sections were digested in 0.4% w/v pepsin (Sigma) in 0.1M HCl at 37° C. Digestion times were determined in optimisation time trials and varied between 20-25 min for human skin, 15-20 min for mouse skin, 10-15 min for mouse spleen controls, and 2-4 min when *in situ* hybridisation was preceded by immunohistochemistry requiring antigen retrieval. Pepsin digestion was followed by incubation in 0.2% glycine (Merck) in double concentration PBS for 5 min to stop digestion. The sections were then fixed in 4% paraformaldehyde (PFA) for 2 minutes, dehydrated through 70%, 95% and 100% ethanol and air-dried. A 1:1 mixture of Cy3-labelled Y chromosome and FITC labelled X-chromosome probes (Star-FISH) was added to the sections and sealed under a glass coverslip with rubber cement. This was followed by hybridisation at 60° C or 80° C for 10 minutes for human and mouse tissue, respectively. Sections were then incubated overnight at 37° C in a sealed humid chamber. Slides were washed in 0.5xSSC in distilled water for 5 min and mounted in VECTASHIELD® Hard Set™ mounting medium containing DAPI (Vector Laboratories).

2.6.5 Immunohistochemistry combined with Y-FISH

Sections were dewaxed, subjected to antigen retrieval as needed and stained using primary antibodies against either pan-cytokeratin, α-SMA, CD34, CD31 or HPV. Stainings were performed using the alkaline phosphatase technique and visualised with Vector Red. After immunocytochemical staining, the Y-FISH protocol was followed, with adjusted digestion times depending on the method used for antigen retrieval.

2.6.6 Microscopy

Fluorescent immmunohistochemical staining and FISH were detected with a fluorescent Olympus BX41 microscope with Smart Capture software. Immuohistochemistry was visualised under conventional or reflected light

(dark-field) conditions (Nikon ME600 with epi-illumination dark-field microscopy) and images were captured using Qimaging software and a micropublisher 5.0 (GO-5-CLR-12) RTV digital camera from Qimaging.

2.7 ENZYME HISTOCHEMISTRY

Enzyme activity of cytochrome c oxidase was tested in experiments outlined in chapter VII. Cytochrome c oxidase consists of several subunits, of which 3 are encoded in the mitochondrial DNA and 4 are encoded in the genomic DNA. Dual enzyme histochemistry for cytochrome c oxidase and succinate dhydrogenase (SDH) was successively used to detect deficiency in cytochrome c oxidase and simultaneously the activity of the nuclear encoded SDH, the presence of which indicates absence of cytochrome c oxidase activity.

2.7.1 Slide preparation

Human skin tissue was snap frozen in liquid nitrogen-cooled isopentane and kept at -80° C. To cut sections, the tissue was mounted in OCT (Bayer Diagnostics) and cut at 20 μm onto membrane-coated P.A.L.M slides (Zeiss) for subsequent laser capture microdissection.

2.7.2 Histochemical assay for cytochrome c oxidase and succinate dehydrogenase

First, sections were incubated in cytochrome c oxidase medium (100 μM cytochrome c oxidase) (Sigma), 4 μM DAB (Sigma) and 20 μg/ml catalase in 0.2 M phosphate buffer, pH 7.0) until enzyme activity was optically detectable by brown staining (approximately 40 min). After three washes in PBS for 5 min each, sections were incubated in SDH incubation medium (130 mM sodium succinate) (Sigma), 200 μM phneazine methosulphate (Sigma), 1 mM sodium azide (Sigma), 1.5 M nitroblue tetrazolium (Sigma) in 0.2 M phosphate buffer, pH 7.0) until a blue colour was detectable

(approximately 45 min). Sections were washed in PBS three times for 5 min and dehydrated in a graded alcohol series of 70%, 95%, and 100%. They were then left to air dry for 1 hr before laser capture microdissection or storage at -20° C.

2.8 MOLECULAR METHODS

2.8.1 DNA extraction

Sections with paraffin-embedded skin tumours from kidney transplant recipients used for experiments outlined in chapter V were dewaxed and scraped off the slides with a sterile needle. Total RNA was extracted with an RNeasy kit (Qiagen) following the manufacturer's protocol. MRNA content was measured with a NanoDrop ND-1000 (Wilmington) spectrophotometer and stored at -20° C. Proteinase K was deactivated by 10 min incubation at 95° C

Frozen sections from skin tumours used in experiments for chapter VII were, after dual histochemistry, subjected to laser capture microdissection using the PALM® laser capture system (Zeiss). DNA from laser-captured areas was extracted using the Picopure™ DNA extraction kit containing proteinase K (Molecular Devices) by overnight incubation at 65°C. Proteinase K was deactivated by 10 min incubation at 95° C.

2.8.2 Quantitative real-time PCR

50ng aliquots of DNA were subjected to qPCR using the Y-quantifiler kit (Quiagen) containing primers specific to a sequence on the sex-determining gene *SRY* on the Y chromosome. The manufacturer's protocol was followed. Thermocycling was carried out on the ABI 7700 Real-Time PCR system (Applied Biosystems), results were analysed using the standard curve method.

2.8.3 Mitochondrial DNA sequencing

To sequence the mitochondrial (mt) genome of microdissected cells, DNA extraction was followed by a two-round amplification, whereby the first round consisted of amplifying 9 fragments spanning the entire mt genome, and the second round consisted of 36 M13-tailed primer pairs to amplify overlapping segments of the first-round products. Individual mutations were confirmed by repeating the 2nd round PCR and re-sequencing the product.

2.8.3.1 Primary PCR reactions

Primer pairs were designed to amplify 9 overlapping segments of the mitochondrial genome, each of approximately 2 kb in size. PCR reactions were carried out in 1x PCR buffer (10mM Tris-HCl (Sigma) pH 8.3, 1.5 mM $MgCl_2$. 50 mM KCl, 0.001%wt/vol gelatine), 0.2 mM dNTPs, 0.6 μM primers, 1 U AmpliTaq Gold DNA Polymerase (Applied Biosystems) and 1 μl cell lysate from microdissected cells. Primers used for the reactions are listed in Table 2.10 (pg.116). Reaction conditions were as follows:

Initial denaturation	*95°C*	*12 min*	*1 cycle*
Denaturation	*95°C*	*45 sec*	
Primer Annealing	*58°C*	*45 sec*	*38 cycles*
Extension	*72°C*	*2 min*	
Final Extension	*72°C*	*8 min*	*1 cycle*

2.8.3.2 Secondary PCR reaction

Primary PCR products were amplified with 36 overlapping primer pairs that span the entire human mt genome, generating fragments between 600-700 bp. Each first round PCR product was amplified by 4 second-round primer pairs, which generated a total of 36 overlapping fragments. Primer pairs used for the reactions were M13-tailed, which resulted in the incorporation of the M13-sequence in the final PCR product. Primers are listed in Table 2.10. PCR reactions were carried out in 1x PCR buffer, using 2 μl of the

previous PCR product as template. The thermocycling procedure was as follows:

Initial denaturation	*95°C*	*12 min*	*1 cycle*
Denaturation	*95°C*	*45 sec*	
Primer Annealing	*58° C*	*45 sec*	*38 cycles*
Extension	*72° C*	*1 min*	
Final Extension	*72° C*	*8 min*	*1 cycle*

2.8.3.3 Agarose gel electrophoresis

To test whether PCR amplification was successful, 5 μl of PCR products were mixed with 1 μl loading dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol) and loaded onto a 1.5% agarose gel in TBE containing ethidium bromide (Merck). A 100 bp ladder (Quiagen) was used as molecular marker. Electrophoresis was carried out for 30 min at 120 V in 1xTBE (0.8 mM Tris acetate, Boric acid, 80 mM, 0.02 mM EDTA). Gels were subjected to UV illumination using a UV transilluminator (UVP Ltd).

2.8.3.4 PCR sequencing

PCR products from successful amplifications were sequenced, if control reactions were negative. PCR products were stripped of any unconsumed dNTPs and primers remaining in the PCR product mixture by incubation of 5 μl PCR product with 2 μl of ExoSAP-IT (USB Corporation) for 15 min at 37°C, followed by inactivation at 80°C for 15 min. Four μl of the resulting PCR product was cycle sequenced using the BigDye® v3.1 terminator cycle sequencing chemistries (Applied Biosystems) and M13 primer. The PCR programme was as follows:

Initial denaturation	*96°C*	*1 min*	*1 cycle*
Denaturation	*96°C*	*20 sec*	*25 cycles*
Primer Annealing	*50°C*	*20 sec*	
Extension	*60°C*	*4 min*	

DNA was purified using the DyeEx 2.0 Spin Kit Purification Columns (Quiagen) and the products were analysed on the ABI 3100 sequencer (Applied Biosystems). Sequences were compared to the revised Cambridge reference sequence using sequence alignment software of the European Molecular Biology Open Software Suite (2000) (EMBOSS).

2.9 STATISTICAL ANALYSES

All data in tables, graphs or text represent the mean values ± SEM. Tests of significance (Prism) were conducted using Mann-Whitney or Kruskal-Wallis analyses with Dunn's post-test for non-parametric data and the Student's two-tailed t-test for parametric data where appropriate. Binomial data were analysed using the Chi-square test. Probability (P) values of ≤ 0.05 were taken as appropriate levels of significance.

Table 2.1

Tumorigenicity of sorted SP and Non-SP cells upon s.c. injection into nude mice

	Cell numbers injected				
	10^2	$1x10^3$	$1x10^4$	$5x10^4$	$1.5x10^5$
HT29					
SP cells	3/5	3/3	1/1	--	--
Non-SP cells	2/5	3/3	1/1	0/1	1/1
Caco2					
SPcells	1/5	1/3	1/1	--	--
Non-SP cells	3/5	2/3	1/1	1/1	0/1
HRA19					
SP	2/5	0/3	0/1	--	--
Non-SP	3/5	0/3	0/1	1/1	0/1
HGT101					
SP	0/5	0/3	0/1	--	--
Non-SP	0/5	0/3	0/1	0/1	0/1

Mice culled after 16 weeks or when tumour burden reached a threshold diameter of 15 mm.

Table 2.2

Injection of sorted cell populations from fresh human colonic adenocarcinoma (groups 1-6) and normal epithelium (groups 7-12) s.c. into nude mice

Groups (n=3)	Sorted cell populations (+cells were injected on the left flank, -cells were injected on the right flank)					
	CD44+	CD44-	CD133+	CD133-	CD44/133+	CD44/133-
Group 1/7	100	100				
Group 2/8	1000	1000				
Group 3/9			100	100		
Group 4/10			1000	1000		
Group 5/11					100	100
Group 6/12					1000	1000

Table 2.3

BM transplantation of HPV 8 mice

Recipient / BM	Wt		HPV	
	No wounding	wounding	No wounding	wouding
HPV	group1	group2	group3	group4
wt	group5	group6	group7	group8

Table 2.4

Lesions developed after kidney transplants included in the study described in chapter V

	Type of lesion	Number of patients affected	Total number of lesions
Malignant	Basal Cell Carcinoma (BCC)	17	48
	Squamous Cell Carcinoma (SCC)	23	47
	Porocarcinoma	3	3
Pre-malignant	Carcinoma *in situ* (CIS)	17	43
	Actinic/Bowenoid Keratosis (AK/BK)	16	26
	Lentigo Maligna	1	1
	Dysplastic Warts	3	3

Table 2.5

Optimal staining conditions as determined for each tested gastrointestinal tumour cell line

Cell line	Hoechst 33342 (μg/ml)	Incubation time (min)
Caco2	6	60
HT29	7.5	60
HGT101	5	60
HRA19b1.1	7.5	90
Col29	7.5	60
Col1	7.5	60
SW480	9	45

Table 2.6

Fluorescent antibodies used throughout the project

Antibody	Fluorescent label	Wavelength of fluorescent signal	Amount	Supplier
CD44	APC (Allophycocyanin)	660/20	10μl/10^6 cells	BD Biosciences
CD133	PE (Phycoerythrin)	575/26	10μl/10^6 cells	Miltenyi Biotec
CD34	PE/Cy7 (Cyanine)	780/60	5μl/10^6 cells	BD Biosciences
CD117	PerCP (Peridinin-chlorophyll-protein Complex)/Cy5.5	695/40	20μl/10^6 cells	BD Biosciences
BCRP1	PE/Cy5	660/20	10μl/10^6 cells	Chemicon
Ki-67	FITC	530/30	20μl(1:100)/ 10^6 cells	BD Bioscences

Table 2.7

Primary antibodies used on cell lines

Antibody	Specificity	Species	Dilution	Antigen Retrieval	Supplier
β-catenin	human	mouse	1:100	N/A	Santa Cruz
CD117	human	mouse	1:40	N/A	Novocastra
CD34	human	mouse	1:25	N/A	DAKO
Chromogranin A	human	mouse	1:50	N/A	DAKO
Hes-1	human	rabbit	1:200	N/A	US Biological
Mdr1	human	mouse	1:200	N/A	Chemicon
Muc2	human	rabbit	1:100	N/A	Santa Cruz
Mucin PR4D4	human	mouse	N/A	N/A	Cancer Research UK
Musashi-1	human	mouse	1:20	N/A	R&D systems
Oct-4	human	mouse	1:100	N/A	Santa Cruz
Villin	human	mouse	1:100	N/A	Serotec

Table 2.8

Primary antibodies used on paraffin-embedded tissue

Antibody	Specificity	Species	Dilution	Antigen Retrieval	Supplier
α-SMA	human/mouse	mouse	1:4000	N/A	Sigma
Chromogranin A	human	mouse	1:50	N/A	DAKO
CD10	human	mouse	1:100	mwv 10'	Abcam
CD133	human	rabbit	1:500	pressure cooking 2', overnight incubation at 4°C	Abcam
CD31	mouse	rat	1:20	mwv in EDTA 10'	Pharmingen
CD34	human	mouse	1:25	mwv 10'	DAKO
CD44	human	mouse	1:100	mwv/pressure cooking 2'	Abcam
CD45	human	mouse	1:200	mwv 10'	DAKO
CD45	mouse	rat	1:20	mwv in EDTA	Pharmingen
Cytochrome c oxidase subunit 1	human	mouse	1:250	mwv 10'	Invitrogen
Cytochrome c oxidase subunit 2	human	mouse	1:250	mwv 10'	Invitrogen
HPV	human	rabbit	1:40	none/overnight incubation at 4°C	Abcam
Ki-67	human/mouse	rabbit	1:200	mwv 20'	Novacastra
Muc2	human	rabbit	1:100	mwv 10'	Santa Cruz
Pan-cytokeratin	human/mouse	mouse	1:25	trypsin 15'	DAKO
Villin	human	mouse	1:100	mwv 10'	Serotec

(mwv = microwaving in boiling sodium citrate buffer, pH 6.0)

Table 2.9

Details of secondary antibodies and species-specific sera used throughout the project

Immunoglobulin/Serum	Specificity	Species	Label	Dilution	Source
Rabbit anti-mouse IgG biotin conjugate	mouse	rabbit	biotin	1:300	DAKO
Swine anti-rabbit biotin conjugate	rabbit	swine	biotin	1:500	DAKO
Goat anti-rabbit biotin conjugate	goat	rabbit	biotin	1:100	Sigma
Goat anti-mouse IgG af555 (FITC) conjugate	mouse	goat	FITC	1:200	Invitrogen
Goat anti-rabbit IgG af488 (Cy3)	rabbit	goat	Cy3	1:1000	Invitrogen
Swine serum				1:25	DAKO
Rabbit Serum				1:25	DAKO
Goat Serum				1:25	DAKO

Table 2.10

1st and 2nd round primers for amplification of the mitochondrial genome

1st Round Primers	Position
AF GCTCACATCACCCCATAAAC	627-646
AR GATTACTCCGGTCTGAACTC	3087-3068
BF ACCAACAAGTCATTATTACCC	2395-2415
BR TGAGGAAATACTTGATGGCAG	4653-4633
CF CCGTCATCTACTCTACCATC	4489-4508
CR GGACGGATCAGACGAAGAG	6468-6450
DF AATACCCATCATAATCGGAGG	6113-6133
DR GGTGATGAGGAATAGTGTAAG	8437-8417
EF AACCACTTTCACCGCTACAC	8128-8147
ER AGTGAGATGGTAAATGCTAG	10516-10487
FF ACTTCACGTCATTATTGGCTC	9821-9841
FR ATAGGAGGAGAATGGGGGATAG	12101-12080
GF ACCCCCCACTATTAACCTACTG	11866-11887
GR GGTAGAATCCGAGTATGTTGG	13924-13904
HF TATTCGCAGGATTTCTCATTAC	13721-13742
HR AGCTTTGGGTGCTAATGGTG	15997-15978
IF CCCATCCTCCATATATCCAAAC	15659-15680
IR GGTTAGTATAGCTTAGTTAAAC	868-847
Second Round Primers	**Positions**
1F TGTAAAACGACGGCCAGTTCACCCTCTAAATCACCAG	721-740
1R CAGGAAACAGCTATGACCGATGGCGGTATATAGGCTGAG	1268-1248
2F TGTAAAACGACGGCCAGTTTAAAACTCAAAGGACCTGGC	1157-1177
2R CAGGAAACAGCTATGACCCTGGTAGTAAGGTGGAGTGGG	1709-1689
3F TGTAAAACGACGGCCAGTAACTTAACTTGACCGCTCTGAG	1650-1671
3R TGTAAAACGACGGCCAGTAACTTAACTTGACCGCTCTGAG	2193-2175
4F TGTAAAACGACGGCCAGTACTGTTAGTCCAAAGAGGAAC	2091-2111
4R CAGGAAACAGCTATGACCTCGTGGAGCCATTCATACAG	2644-2625
5F TGTAAAACGACGGCCAGTCAGTGACACATGTTTAACGGC	2549-2569
5R CAGGAAACAGCTATGACCGATTACTCCGGTCTGAACTC	3087-3068
6F TGTAAAACGACGGCCAGTCAGCCGCTATTAAAGGTTCG	3017-3036
6R CAGGAAACAGCTATGACCGGAGGGGGGTTCATAGTAG	3374- 3356
7F TGTAAAACGACGGCCAGTCCTTAGCTCTCACCATCGC	3533-3351
7R CAGGAAACAGCTATGACCAGAGTGCGTCATATGTTGTTC	4057-4037

8F TGTAAAACGACGGCCAGTAATAAACACCCTCACCACTAC	4005-4025
8R CAGGAAACAGCTATGACCGTTTATTTCTAGGCCTACTCAG	4577-4556
9F TGTAAAACGACGGCCAGTACACTCATCACAGCGCTAAG	4518-4537
9R CAGGAAACAGCTATGACCGATTTTGCGTAGCTGGGTTTG	5003-4983
10F TGTAAAACGACGGCCAGTTCCATCATAGCAGGCAGTTG	4950-4969
10R CAGGAAACAGCTATGACCTGTAGGAGTAGCGTGGTAAGG	5481-5462
11F TGTAAAACGACGGCCAGTACCTCAATCACACTACTCCC	5367-5386
11R CAGGAAACAGCTATGACCTAGTCAACGGTCGGCGAAC	5924-5906
12F TGTAAAACGACGGCCAGTCACTCAGCCATTTTACCTCAC	5875-5895
12R CAGGAAACAGCTATGACCATGGCAGGGGGTTTTATATTG	6430-6410
13F TGTAAAACGACGGCCAGTTTAGGGGCCATCAATTTCATC	6378-6398
13R CAGGAAACAGCTATGACCAAGAAAGATGAATCCTAGGGC	6944-6924
14F TGTAAAACGACGGCCAGTATTTAGCTGACTCGCCACAC	6863-6882
14R CAGGAAACAGCTATGACCCATCCATATAGTCACTCCAGG	7396-7376
15F TGTAAAACGACGGCCAGTGGCTCATTCATTTCTCTAACAG	7272-7293
15R CAGGAAACAGCTATGACCGGCAGGATAGTTCAGACGG	7791-7773
16F TGTAAAACGACGGCCAGTTAACATCTCAGACGCTCAGG	7744-7763
16R CAGGAAACAGCTATGACCTACAGTGGGCTCTAGAGGG	8301-8283
17F TGTAAAACGACGGCCAGTACAGTTTCATGCCCATCGTC	8196-8215
17R CAGGAAACAGCTATGACCGTATAAGAGATCAGGTTCGTC	8740-8720
18F TGTAAAACGACGGCCAGTACCACCCAACAATGACTAATC	8656-8676
18R CAGGAAACAGCTATGACCGTTGTCGTGCAGGTAGAGG	9201-9183
19F TGTAAAACGACGGCCAGTATCCTAGAAATCGCTGTCGC	9127-9146
19R CAGGAAACAGCTATGACCATTAGACTATGGTGAGCTCAG	9661-9641
20F TGTAAAACGACGGCCAGTCATCCGTATTACTCGCATCAG	9607-9627
20R CAGGAAACAGCTATGACCTAGCCGTTGAGTTGTGGTAG	10147-10128
21F TGTAAAACGACGGCCAGTCAACACCCTCCTAGCCTTAC	10085-10104
21R CAGGAAACAGCTATGACCAGGCACAATATTGGCTAAGAG	10649-10629
22F TGTAAAACGACGGCCAGTATCGCTCACACCTCATATCC	10534-10553
22R CAGGAAACAGCTATGACCATGATTAGTTCTGTGGCTGTG	11109-11089
23F TGTAAAACGACGGCCAGTCTAATCTCCCTACAAATCTCC	11054-11074
23R CAGGAAACAGCTATGACCTAGGTCTGTTTGTCGTAGGC	11605-11586
24F TGTAAAACGACGGCCAGTTCCTTGTACTATCCCTATGAG	11541-11561
24R CAGGAAACAGCTATGACCCGTGTGAATGAGGGTTTTATG	12054-12034
25F TGTAAAACGACGGCCAGTACAATGGGGCTCACTCACC	12001-12019
25R CAGGAAACAGCTATGACCGTGGCTCAGTGTCAGTTCG	12545-12527
26F TGTAAAACGACGGCCAGTCATGTGCCTAGACCAAGAAG	12498-12517
26R CAGGAAACAGCTATGACCCTGATTTGCCTGCTGCTGC	13009-12991
27F TGTAAAACGACGGCCAGTGCCCTTCTAAACGCTAATCC	12940-12959
27R CAGGAAACAGCTATGACCGGGAGGTTGAAGTGAGAGG	13453-13435

28F TGTAAAACGACGGCCAGTCGGGTCCATCATCCACAAC	13365-13383
28R CAGGAAACAGCTATGACCGTTAGGTAGTTGAGGTCTAGG	13859-13839
29F TGTAAAACGACGGCCAGTACCTAAAACTCACAGCCCTC	13790-13809
29R CAGGAAACAGCTATGACCAGGATTGGTGCTGTGGGTG	14374-14356
30F TGTAAAACGACGGCCAGTCAACCACCACCCCATCATAC	14331-14350
30R CAGGAAACAGCTATGACCAAGGAGTGAGCCGAAGTTTC	14857-14838
31F TGTAAAACGACGGCCAGTATTCATCGACCTCCCCACC	14797-14815
31R CAGGAAACAGCTATGACCGGTTGTTTGATCCCGTTTCG	15368-15349
32F TGTAAAACGACGGCCAGTAGCCCTAGCAACACTCCAC	15316-15334
32R CAGGAAACAGCTATGACCTACAAGGACAGGCCCATTTG	15896-15877
D1F TGTAAAACGACGGCCAGTATCGGAGGACAACCAGTAAG	15758-15777
D1R CAGGAAACAGCTATGACCGTGGGTAGGTTTGTTGGTATC	16294-16274
D2F TGTAAAACGACGGCCAGTCTCAACTATCACACATCAACTG	16223-16244
D2R CAGGAAACAGCTATGACCAGATACTGCGACATAGGGTG	129-110
D3F TGTAAAACGACGGCCAGTCACCCTATTAACCACTCACG	15-34
D3R CAGGAAACAGCTATGACCCTGGTTAGGCTGGTGTTAGG	389-370
D4F TGTAAAACGACGGCCAGTGCCACAGCACTTAAACACATC	323-343
D4R CAGGAAACAGCTATGACCTGCTGCGTGCTTGATGCTTG	771-752

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 CULTURE

Dulbecco's modified Eagle's medium (CRUK), was supplemented with 100 IU/L penicillin (Gibco BRL) and 100 μg/L streptomycin (Gibco BRL) and 10% FCS. Medium was stored at 4°C until use

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.

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 1 L total volume.

Sodium citrate buffer (pH 6.0)

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

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%

TBE Buffer (10x)

NaOH	1g
Tris Base	108g
Boric Acid	55g
EDTA	7.4g
Distilled H_2O	1L

SUPPLIERS AND DISTRIBUTORS

Applied Biosystems,	Foster City, CA, USA
Abcam	Cambridge, UK
Bayer Diagnostics	Berkshire, UK
BD Biosciences	New Jersey, USA
BD Falcon	Franklin Lake, OH, USA
Beckman Coulter	Fullerton, CA, USA
Becton Dickinson	Franklin Lakes, OH, USA
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Cambio Ltd.	Cambridge, UK
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Chemicon	Temecula, CA, USA
Cis-Bio International	Bagnols, France
DAKO	Cambridge, UK.
Dakocytomation	Glostrup, Denmark
Euro-Diagnostica	Malmo, Sweden
Invitrogen	Carlsbad, CA, USA
Gibco BRL/Life Technologies Ltd.	Renfrewshire, UK.
LabTek™	Brendale, Australia
Merck and Co. Inc.	New Jersey, USA
Millipore	Watford, UK
Miltenyi Biotec	Bergisch-Gladbach, Germany
Molecular Probes	Leiden, Netherlands
New England Biolabs (NEB)	New York, USA
Novocastra	Newcastle upon Tyne, UK
PAA Laboratories GmbH	Pasching, Austrua
Molecular Devices	Sunnyvale, CA, USA
Prism/GraphPad Software	San Diego, CA, USA
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Quiagen	Hilden, Germany
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Sigma Chemical Co.,	Dorset, UK
Star-FISH, Cambio Ltd.	Cambridge, UK
Treestar	San Carlos, CA
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USB Corporation	Cleveland, OH, USA
UVP	Upland, CA, USA
Vector Laboratories	Burlingame, CA, USA

CHAPTER III

SIDE POPULATIONS OF HUMAN GASTROINTESTINAL CANCER CELL LINES ARE NOT ENRICHED IN STEM CELLS

3.1 INTRODUCTION

It is well known that stem cells are involved in disease processes. Particular attention has been paid to their involvement in cancer origin and development and the research community focuses with substantial effort on the search for potential markers that could distinguish stem cells and cancer stem cells.

The Side Population (SP) phenotype has been suggested as a universal stem cell marker. It is defined by the ability to efflux the nucleic acid dye Hoechst 33342; attributed to the activity of ABC-membrane transporter proteins, which are also thought to equip cancer stem cells with a resistance to lipophilic drugs and are blockable by efflux pump inhibitors and chemosensitising agents such as verapamil (Goodell *et al.* 1997), reserpine (Zhou *et al.* 2001) and fumitremorgen C (Budak *et al.* 2005). SPs have been found in several human tissues (Alvi *et al.* 2003, Asakura *et al.* 2002, Bhattacharya *et al.* 2003, Poliakova *et al.* 2004, Tabatabai *et al.* 2005), cancers (Grichnik 2006, Hirschmann-Jax *et al.* 2004, Szotek *et al.* 2006), and cancer cell lines (Chiba *et al.* 2006, Dekaney *et al.* 2005), and in some reports have been shown to display stem cell activity.

However, few studies have directly compared SP and non-SP cells, the results obtained have been varied and contradictory and recently, several groups have not been able to confirm the stem cell-enrichment of SPs in various tissues (Mitsutake *et al.* 2007, Morita *et al.* 2006). In the

gastrointestinal system, SPs have not yet been examined for stem cell enrichment.

Here, we examine various human gastrointestinal cancer cell lines for the presence of the SP phenotype and directly compare phenotypic and behavioural characteristics of Non-SP and SP cell fractions with regard to stem cell-typical features. We assess clonogenic ability *in vitro*, drug resistance *in vitro*, tumourigenity *in vivo* and differentiation potential both, *in vitro* and *in vivo*. Additionally, we assess both cell fractions phenotypically before and after culture *in vitro,* and after xenograft tumour formation for the expression of putative stem cell markers and the presence of the SP phenotype. Moreover, we test for differences in proliferation capacity, measured by the expression of Ki-67 and also compare the expression of the BCRP1 (ABCG2) transmembrane transporter protein, which is thought to be responsible for the SP phenotype (Patrawala *et al.* 2005, Zhou *et al.* 2001); (Zhou *et al.* 2002).

3.2 AIMS

To date, the origin and mechanism of disease development in gastrointestinal cancers is not entirely clear. Although a cell hierarchy is known to exist in these cancers, no markers for cancer stem cells in gastrointestinal cancers has been identified. This project aimed to identify and characterise SPs in gastrointestinal cancer cell lines and to elucidate whether the SP phenotype can serve as a cancer stem cell marker. We sought to directly compare sorted side population and non-side population cells with respect to phenotypical and behavioural stem cell-typical characteristics such as expression of surface markers, the capabilities to self-renew and proliferate and for multipotential lineage differentiation.

3.3 METHODS

All methods are described in full in Chapter II.

3.3.1 Cell culture

The human gastrointestinal cancer cell lines used for our experiments were of colorectal (HT29, HRA19a1.1 [doubly cloned from HRA19 (Kirkland *et al.* 1994)], Col1, Col29 [sublines of HCA7 (Marsh *et al.* 1993)], SW480, SW1222) and gastric (HGT101 (Laboisse *et al.* 1982)) origin. The cell lines were incubated and maintained at 37°C in a humidified atmosphere containing 8% CO_2.

3.3.2 Side population analysis

To identify the presence of SPs, the protocol developed by Goodell *et al.*, (Goodell *et al.* 1997) was largely followed. Reserpine (100 μmol/L) was used to verify the SP phenotype as described by Zhou *et al.* (Zhou *et al.* 2001). Dead cells were excluded by labelling with propidium iodide (PI). The stained cells were analysed using a LSR II Flow cytometer (BD Biosciences, San Jose, USA).

Three cell lines (HT29, Caco2, HGT101) were selected for further experiments based on their origin and growth characteristics. *In vivo* experiments were carried out with 4 cell lines, using HRA19a1.1 cells in addition to the three above mentioned cell lines.

3.3.3 Fluorescence-activated cell sorting

For sorting of SPs, non-SPs and whole populations, cells were stained according to the optimal conditions determined above for each of the selected cell lines. The SP was defined as the Hoechst 33342-low tail population, blockable by the addition of reserpine. Cells retaining Hoechst 33342 fluorescence were determined as the non-SP population. Cell sorting was carried out on a MoFlo™ High Performance Cell Sorter

(Dakocytomation, Glostrup, Denmark) with excitation and emission wavelengths as stated in chapter II.

Doublets and PI-stained dead cells were electronically gated out. Inhibition of the SP phenotype by reserpine was used as guidance for drawing of appropriate sorting gates.

3.3.4 Multicolour immunofluorescence flow cytometric analysis

To determine whether SP cells differ from non-SP cells in surface marker expression, we analysed the selected cell lines (HGT101, HT29 and Caco2) for expression of the putative stem cell markers CD34, CD44, CD117 (c-KIT), CD133 (prominin1) and BCRP1 (ABCG2). Cells were first stained with Hoechst 33342 under previously determined optimal conditions and then stained with directly labelled fluorescent antibodies against CD34, CD44, CD117, CD133, and BCRP1.

The stained cells were analysed using a LSR II Flow cytometer. Data were analyzed using FloJo computer software (Treestar, San Carlos, CA).

3.3.5 Clonogenic assays

To analyse clonogenic and morphological properties of SP and non-SP cells of HGT101, HT29 and Caco2 cell lines, single cells of both fractions were sorted into 96-well plates and cultured. After 3 weeks, cells were fixed in 4% PFA and cloning efficiency was measured to indicate the percentage of cells that initiated a clone. Size and morphology of the clones was assessed, whereby the different morphological behaviour displayed by Caco2 clones was taken into account by a classification system separate from HT29 and HGT101 cells. Experiments were performed in triplicate, and repeated twice.

3.3.6 Re-analysis of cultured cell fractions

To examine long-term behaviour of sorted SP and non-SP cells, cells were seeded at clonal density in T25 culture flasks (Becton Dickinson, Franklin Lakes, USA) and maintained in the culture conditions described above.

3.3.7 Differentiation assays

Purified SP and non-SP cells were plated at $1x10^3$ cells/chamber in plastic 8-chamber slides (LabTek^{TM,} Brendale, Australia). After reaching confluency, differentiation was triggered by sodium butyrate. Cells were maintained and fixed as described before.

3.3.8 Immunohistochemistry

The fixed colonies derived from the clonogenic assay described above were stained with antibodies against putative stem cell markers, namely β-catenin, Oct-4, Hes-1, Musashi-1, CD117, CD34, Muc2, Villin, Chromogranin A and Mucin PR4D4.

3.3.9 Proliferation capacity measured by expression of Ki-67

To test the proliferating ability of SP and non-SPs, the sorted cell populations were fixed in suspension in icecold 70% methanol, washed and incubated with a directly conjugated fluorescent antibody against Ki-67. Subsequently, emitted fluorescence signals of the stained cells were analysed on the LSR II Flow cytometer. The percentage distribution of cells among the different cell stages was determined by Watson Pragmatic analysis.

3.3.10 Resistance to 5-fluorouracil (5-FU)

Drug resistance of the different cell fractions was analysed by subjecting cells to the chemotherapeutic agent 5-FU. Cells were incubated in medium supplemented with 5-FU for 3 weeks. To test whether any cell population

was more susceptible to the toxic effects of 5-FU, the cells were analysed previous to and following the incubation period for the SP phenotype and expression of CD133, CD44, CD117 and BCRP1 by multicolour fluorescence analysis as outlined in 3.3.4.

3.3.11 Tumour-forming ability *in vivo* (xenografts)

Xenograft experiments were performed using 4 selected cell lines (HT29, HGT101, Caco2, HRA19a1.1). Sorted SP or non-SP population cells were injected s.c. into 4-8 week-old nude mice (Table 3.3, pg.152). Tumour development was monitored starting from the second week, and then measured weekly. All animals were culled 16 weeks after injection or when tumour size passed the limit of 15 mm in diameter. Tumours were harvested and processed as explained in section 2.4.2.

3.3.12 Statistical analysis

All data in tables, graphs or text represent the mean values ± SEM. Tests of significance (Prism, GraphPad, San Diego) were conducted using the Kruskal-Wallis analysis with Dunn's post-test for non-parametric data and the Student's two-tailed t-test for parametric data where appropriate. A probability (P) value of ≤ 0.05 was taken as an appropriate level of significance.

3.4 RESULTS

3.4.1 Prevalence of SP phenotype in human gastrointestinal cancer cell lines

The SP phenotype as defined by low Hoechst 33342 blue and red fluorescence intensity was detected in all tested human gastrointestinal carcinoma cell lines, ranging between 1.3-17% of cells (Figure 3.1, pg.140). Maximal inhibition of the SP-phenotype in reserpine control samples verified the SPs and indicated optimal staining conditions for each cell line (Table 3.1, pg.150).

3.4.2 Cell surface phenotype

To elucidate potential phenotypic differences between SP cells and non-SP cells, we analysed both populations of cultured Caco2, HT29 and HGT101 cell lines for the presence of putative stem cell-specific surface markers by flow-cytometric analysis. The overall staining patterns were similar between cell lines (Table 3.2, left column, pg.151). The vast majority of cells from all cell lines expressed CD44 and CD133, with expression levels ranging between 87-100% and 76-98%, respectively. Expression of CD117 (cKit), a marker of progenitor cells in several human tissues (reviewed in (Miettinen *et al.* 2005)) was barely detectable in Caco2 cells, whereas its expression levels in HT29 and HGT101 reached 1.2±0.3% and 4.1±1.6%, respectively. CD34 expression showed the largest variation, and interestingly, was the only stem cell marker that was significantly different in its levels of expression between SP and non-SP fractions. At the time of sorting, the SPs were virtually devoid of CD34 expression in HGT101 and HT29, whereas their non-SP counterparts reached expression levels of 2.5% and 0.6%. Interestingly, between SP and non-SP fractions, we could not detect a significant difference in expression of BCRP1, the ABC-transporter allegedly responsible for the SP phenotype, which ranged between 1.6-4.1% depending on the cell line.

3.4.3 *In vitro* characteristics of SP and non-SP cells

The clonogenic efficiencies of SP and non-SP cells from HT29, HGT101 and Caco2 were similar (Figure 3.2a,A, pg.141), all differences were not statistically significant. This was somewhat surprising, considering that the predicted behaviour of precursor cells would include a higher capacity for self-renewal. HT29 cells displayed the highest clonogenic potential of 66±3.7% and 70±3.3% for SP and non-SP cells, respectively. HGT101 SP cells and non-SP cells displayed colony-forming efficiencies of 31±3.2% and 40±4.7% respectively, whereas Caco2 cells were least efficient with 9±3.7% for SP cells and 15±3.6% for non-SP cells. The clone sizes were

similar in SP and non-SP derived colonies, indicating that there are no differences in doubling time.

Within each cell line, we could not detect significant differences in the morphologies of the clones generated by SP and non-SP cells. The colonies formed by Caco2 cells were morphologically distinct from those originating from HGT101 and HT29 cells and thus categorised independently (Figure 3.2a, pg.141). HGT101 and HT29 cells formed colonies of 4 different morphologies and were categorised into very small (<50 cells) (Figure 3.2a,B), tight colonies displaying clear borders (holoclones) (Figure 3.2a,C), dispersed (meroclones) (Figure 3.2a,D) and intermediate containing areas of tightly packed and dispersed cells with indefinite borders (paraclones) (Figure 3.2a,E). Caco2 cells typically formed holoclones, which were categorised by size as small (<50 cells) (Figure 3.2a,F), intermediate (Figure 3.2a,G), and large (Figure 3.2a,H). After 3 weeks in culture, HGT101 cells predominantly formed paraclones with no significant difference between SPs and non-SPs (Figure 3.2a,I), whereas HT29 cells mainly formed meroclones with a frequency of 43±2.7% in SP colonies and 37±3.1% in non-SP colonies (Figure 3.2a,J). Only 16±4.5% of HT29 SP colonies and 22±4.8% of HT29 non-SP colonies were meroclones. Both populations of HT29 and HGT101 colonies displayed similar percentages of very small colonies and holoclones, ranging between 9-13% and 24-30%, respectively. The majority of colonies derived from Caco2 cells were classified as large; 50±11% of SP-derived colonies and 52±9.6% of non-SP derived colonies were large, whereas only a few remained very small (Figure 3.2a,K). The differences in morphology type-distribution and colony sizes between SP and non-SP colonies were not significant for any of the cell lines.

Immunophenotypic analysis of the clones generated by SP and non-SP cells did not show differences in the expression of putative stem cell markers in any of the cell lines. Figure 3.2b displays examples of the staining profile of Caco2 cells. After 3-weeks in culture, all clones were

strongly positive for CD133, β-catenin, HES-1, and the putative intestinal stem cell marker Musashi-1, whereas expression of CD117 and OCT4 were not detected on either SP-originated or non-SP-originated colonies. CD34-expression, as well as sporadic MDR-1-expressing cells were detected on clones generated from either cell fraction.

3.4.4 Re-analysis of SP phenotype

Re-analysis of Caco2, HT29 and HGT101 SP and non-SP cells grown for 3 weeks showed the presence of the SP phenotype in both SP and non-SP derived cultures, indicating that both cell fractions have the ability to give rise to the other (Figure 3.3, pg. 143). However, after 3-weeks of culture, the SP fractions observed in the SP-derived culture were larger than the original SP-percentage in any given cell line, and similarly, the SP fraction appearing in the non-SP derived cells was smaller than the original SP phenotype. These results suggest that the cultures, starting from a pure SP and non-SP population, have not had enough time to reach equilibrium between SP and non-SP populations. This is also supported by the fact that cell lines with faster doubling times (HGT101,HT29) were closer to reaching equilibrium after 3-weeks culture than slower growing cell lines (Caco2).

3.4.5 Differentiation potential *in vitro* and *in vivo*

Subjecting both SP and non-SP cells to differentiation-promoting culture conditions using sodium butyrate for 72 hours resulted in successful mucous cell (Figure 3.4A,D, pg.144), enterocyte cell (Figure 3.4B,E) and endocrine cell (Figure 3.4C,F) differentiation by Caco2 cells. In accordance with the known profiles of HT29 and HGT101 cell lines, these cells were capable of enterocytic and mucous cell differentiation, but endocrine cells were only seen in Caco2 and HRA19 non-SP and SP cells. These results confirm the multilineage potential *in vitro* for both cell fractions.

The capability for multipotential differentiation of both SP and non-SP cells was also confirmed *in vivo* by immunocytochemistry on xenograft tumours

removed after reaching the threshold diameter of 1.5 cm. Tumours generated from both cell fractions of Caco2 and HRA19 cells were capable of differentiation into all intestinal epithelial cell types. As shown in Figure 3.4 G-L, xenograft tumours derived from Caco2 SP and non-SP cells show differentiation into mucous cells (Figure 3.4G,J) enterocytes (Figure 3.4H,K) and endocrine cells (Figure 3.4I,L). HT29 cells displayed their typical differentiation profile in tumours generated from non-SP and SP cells, forming enterocytes and mucous cells, but not endocrine cells.

3.4.6 Proliferation capacity measured by expression of Ki-67

Ki-67 is expressed by proliferative cells and used as a marker for cells from late G-phase to mitosis. We failed to detect a significant difference in expression levels of Ki-67 by FAC analysis of fluorescently labelled sorted SP and non-SP cells (Figure 3.5, pg.145). The percentages of positive cells were 3.88% and 4.2% for non-SP and SP cells, respectively. Thus, both cell populations show similar proportions of proliferating and quiescent cells, attributes that are often used to differentiate stem cells from transit amplifying and more mature somatic cells.

3.4.7 Drug resistance to 5-fluorouracil (5-FU)

Subjecting cells to the chemotherapeutic agent 5-FU did not selectively compromise any of the cell populations we tested, including cell populations expressing CD133, CD44, CD117, BCRP, as well as the SP and non-SP (Figure 3.6, pg.146). The population fractions before and after culturing of the cell lines in the presence of 5-FU were not different with statistical significance. Fractions of BCRP1-positive and CD117-positive cells were below 2% before and after treatment with 5-FU, whereas the CD133 and CD44-expressing cell populations displayed constant fractions of over 80%. None of these populations, including the SP showed an elevated drug resistance, which would be typical for a stem cell-enriched population. This indicates that compared to each other, none of these populations is particularly enriched in stem cells.

3.4.8 *In vivo* characteristics of SP and non-SP cells

Subcutaneous injection of varying numbers of freshly-sorted SP and non-SP cells into nude mice showed that both SP and non-SP cell fractions of Caco2, HRA19 and HT29 cells have an equal tumorigenic potential when analysed at 16 weeks after injection or when tumour burden reached a threshold diameter of 1.5 cm. Non-SP cells formed tumours of the same size and latency as SP cells (Table 3.3, pg.152). HGT101 cells however, did not display tumorigenicity *in vivo*, even at the maximum cell dose of 150,000 cells per injection.

As few as 100 sorted cells from HT29, Caco2, and HRA19a1.1 cell lines were capable of tumour formation (Table 3.3, pg.152). There was no significant difference between SP and non-SP cells in the number of tumours generated, nor in tumour size or the latency of tumour appearance. Consistent with their high *in vitro* proliferative activity, HT29 cells were most tumorigenic. The first signs of tumour growth appeared as early as 4 weeks after injection, and tumour growth was rapid with some HT29 SP and non-SP derived tumours reaching the threshold diameter of 15mm by week 10. All tumorigenic cell lines (Caco2, HRA19a1.1, HT29) gave rise to adenocarcinomas resembling the original tumours. There were no morphological or histological differences between SP and non-SP generated tumours (Figure 3.7, pg.147).

Fluorescence-activated cell analysis of the cells extracted from xenograft tumours revealed SPs and non-SPs in all tumours, regardless of whether they originated from SP or non-SP cells, again indicating that both cell fractions are capable of giving rise to the other (Figure 3.8, pg.148). Fluorescent immunophenotyping of the harvested tumour cells by multicolour analysis showed expression profiles similar to the immunophenotypes of the cell fractions prior to FAC-sorting (Table 3.2, right column; Figure 3.8). As before xenografting, the vast majority of both SP and non-SP cells from both cell lines expressed CD44. Moreover, in the Caco2 populations, the CD44-expressing fraction of cells increased with

xenografting, approaching levels of 100%. Similar to the initial profile, a substantial proportion of both HT29 and Caco2 cells also expressed CD133 after xenografting; however, the CD133-expressing population of both SP and non-SP derived HT29 populations was significantly smaller than its original expression prior to grafting. Numbers of CD117 and BCRP1-expressing cells increased slightly on Caco2 populations, although they were reduced on the HT29 cells extracted from the xenograft tumours. The most striking difference between pre-and post-xenograft phenotypes of SP and non-SP cells was the large increase in CD34-expressing cells. Whereas, CD34-expression was barely detectable in the SP fractions at the time of sorting, a substantial fraction, 22.6±3.9% and 47.0±3.7% of Caco2 and HT29 cells respectively, expressed CD34 after tumour formation *in vivo*. Importantly, as we observed an increase in CD34-positive cells *in vitro* as well as *in vivo*, any chance that this may simply be an increase in endothelial cells within the tumours can be ruled out. All other differences in surface marker expression between SP and non-SP fractions after xenografting were not significant for both cell lines (Table 3.2, pg.151).

3. 5 DISCUSSION

3.5.1 Stem cells in normal and cancerous gastrointestinal epithelia

It has been reported that the SP phenotype comprises a cell population highly enriched in stem cells in several adult tissues and human cancers (Srour *et al.* 1993, Asakura *et al.* 2002, Alvi *et al.* 2003, Bhattacharya *et al.* 2003, Chiba *et al.* 2006, Dekaney *et al.* 2005, Morita *et al.* 2006, Szotek *et al.* 2006). However, many of these studies have neglected to directly compare the behavioural characteristics of the SPs and non-SPs derived from the same founding cell population. Moreover, as a large proportion of this research was performed on murine tissues, evidence about the human counterparts is limited. Taken together, these issues raise some doubts about the hypothesis of the SP as a universal stem cell marker. In addition, recent accounts have challenged the notion of SPs as a useful method to identify stem cells (Morita *et al.* 2006, Mitsutake *et al.* 2007).

It is known unequivocally that the gastrointestinal epithelium contains stem cells (reviewed in (Leedham *et al.* 2005)) and it has been proposed that stem cells exist in tumour populations, which may sustain their aggressive biology *in vivo*. However, attempts to isolate normal or tumour stem cells have to date only yielded limited success. Recently, two groups have found that a CD133-positive population to be stem cell-enriched in the human colon, but additional markers still need to be identified to obtain a stem cell population with higher purity. It was our aim to investigate whether the SP phenotype could prove useful for the identification of gastrointestinal cancer stem cells. To accomplish this, we not only sought to determine typical stem-cell behaviour, but also to elucidate whether SP cells can be identified by differential expression of potential stem cell markers.

3.5.2 Side Populations in gastrointestinal cancer cell lines

In the present study, we show that the SP phenotype exists in all of the 7 human gastrointestinal cancer cell lines tested. Six cell lines were derived from colorectal carcinomas and adenocarcinomas. One gastric adenocarcinoma-derived cell line (HGT101) was also found to contain reserpine-sensitive SP cells. Although the SP sizes in our study were significantly higher than SP fractions reported in bone marrow and other primary tissues (Goodell *et al.* 1996, Alvi *et al.* 2003, Bhattacharya *et al.* 2003, Grichnik 2006), they were comparable to the somewhat higher SP-fractions reported in the literature of cells maintained in culture, including cancer cell lines (Hirschmann-Jax *et al.* 2004, Chiba *et al.* 2006, Haraguchi *et al.* 2006). It has to be noted that SP sizes are not correlated to doubling times of the cell lines studied. For example, HGT101 and HT29, the cell lines with shortest doubling time had comparable SP fraction sizes to that of Caco2, a cell line with considerably slower doubling time.

3.5.3 Stem cell-typical characteristics of SP and non-SP cells

We tested for stem cell-typical behaviour, such as the capability for proliferation and self-renewal by single-cell clonogenic assays *in vitro* and tumorigenicity of cells injected into nude mice at progressive dilutions *in vivo*. We also examined the output phenotypes in differentiation-promoting culture conditions *in vitro* and by histological and immunohistochemical examination of xenograft tumours. Additionally, we examined the expression of putative stem cell markers before and after culturing the sorted cell fractions.

Interestingly, non-SP cells displayed not only a similar clonogenic potential *in vitro* as SP cells, but they were also able to form xenograft tumours at limiting dilution with the same size and latency as those derived from SP cells. The possibility of these results representing an artefact due to the effect of Hoechst staining on cell viability was excluded, since single-cell sorted control cells that were not stained with Hoechst 33342 showed the same clonogenicity as Hoechst-stained cells (Figure 3.9, pg.149). If clonogenic potential and tumorigenicity are valid tests for self-renewal and proliferation, these results indicate that non-SP cells contain a similar number of cells with stem cell characteristics as SP cells. Another feature typical for stem cells is their non-cycling, quiescent state. However, we could not detect any difference in the cell cycle status of the populations by measuring the expression of Ki-67, a marker of proliferating cells.

None of our experiments demonstrated any behavioural differences between SP and non-SP cells. Sorted single cells from both populations gave rise to clones of heterogeneous morphologies, as would be expected if multipotent stem cells are contained in each population. Both cell fractions showed the capability for multipotential differentiation *in vitro* and *in vivo,* since enterocytes and mucous cells were detected in SP and non-SP-derived cultures and xenograft tumours of all tested cell lines. The similar histopathological findings of adenocarcinomas formed from both cell

fractions serves as additional evidence for the similar behavioural potential of SP and non-SP cells.

When cultures derived from non-SP and SP cells were re-analysed 3 weeks later, a reserpine-sensitive SP phenotype, as well as a non-SP fraction was observed in cultures originating from both populations. The capability to give rise to the other cell fraction suggests that the populations are interchangeable and provides additional evidence for the presence of immature cells in both populations. Cancer stem cells are discriminated by their heightened resistance to drugs, attributable to the elevated expression of lipophilic multidrug efflux pumps. We reasoned that the exposure to 5-FU, a lipophilic chemotherapeutic agent would selectively compromise the population containing the fewest stem cells. However, after 3-week culture, both the SP and the non-SP appeared in fractions resembling the original SP profile, indicating that no population is enriched for drug-resistant stem cells. Moreover, we showed that 5-FU also did not have an adverse effect on the fractions expressing the putative stem cell surface markers CD133, CD44, CD117, BCRP1. This confirms that these markers are legitimate candidate markers for stem cells since any change in expression profile would indicate a population's vulnerability to the toxic effects of the chemotherapeutic agent.

The only difference we could detect between non-SP and SP cells in our experiments was of a phenotypical nature. Quantifying the expression of CD34, a surface marker whose presence (Srour *et al.* 1993, Krause *et al.* 1994, Morel *et al.* 1996), as well as absence (Cheng *et al.* 1996, Goodell *et al.* 1997), has been associated with stem cells in various tissues, we noticed that non-SP cells expressed CD34, whereas the SP population was CD34-negative. However, after xenografting of sorted SP and non-SP cell fractions, a significant CD34-expression was shown in a subset of SP and non-SPs derived from HT29 and Caco2 cells. This indicates that the CD34-negative population may give rise to CD34 expressing cells. Expression of all other tested putative stem cell markers did not differ between the non-SP and SPs before or after culture or xenografting.

Controversy exists about the relationship between ABCG2 expression and the SP phenotype. In accordance with recent reports (Patrawala *et al.* 2005, Morita *et al.* 2006), we could not support a correlation between the SP phenotype and expression of BCRP1. Expression was identical in non-SP and SP cells before and after culture, indicating that other membrane transporters must play a role in conferring the ability to efflux Hoechst dye.

3.6 CONCLUSION

Our results show that non-SP cells derived from multiple human gastrointestinal cell lines display both progenitor and stem cell characteristics, such as self-renewal, extensive proliferation and differentiation potential, to a similar extent as the SP population. Although the SP fraction clearly contains stem cells, our data indicate that it does not exclusively contain stem cells. Clearly, a substantial portion of stem and progenitor cells, as defined operationally by our experiments, is also contained in the non-SP population. Further research is necessary to isolate stem cells from either population and to elucidate potential behavioural differences that are conferred by the explicit CD34-negativity in the SP population. CD34-negativity has been repeatedly reported to be a marker of immature cells (Krause *et al.* 1994, Morel *et al.* 1996, Osawa *et al.* 1996, Huss 2000). However, although this could indicate that the SP population is enriched in immature cells, we found that the non-SP fraction was proportionately enriched in cells with stem cell-behaviour. Thus, the SP phenotype does not seem to be useful for isolation, purification or characterisation of stem or progenitor cells in gastrointestinal cell lines as defined by clonogenicity, tumorigenity and multilineage differentiation potential.

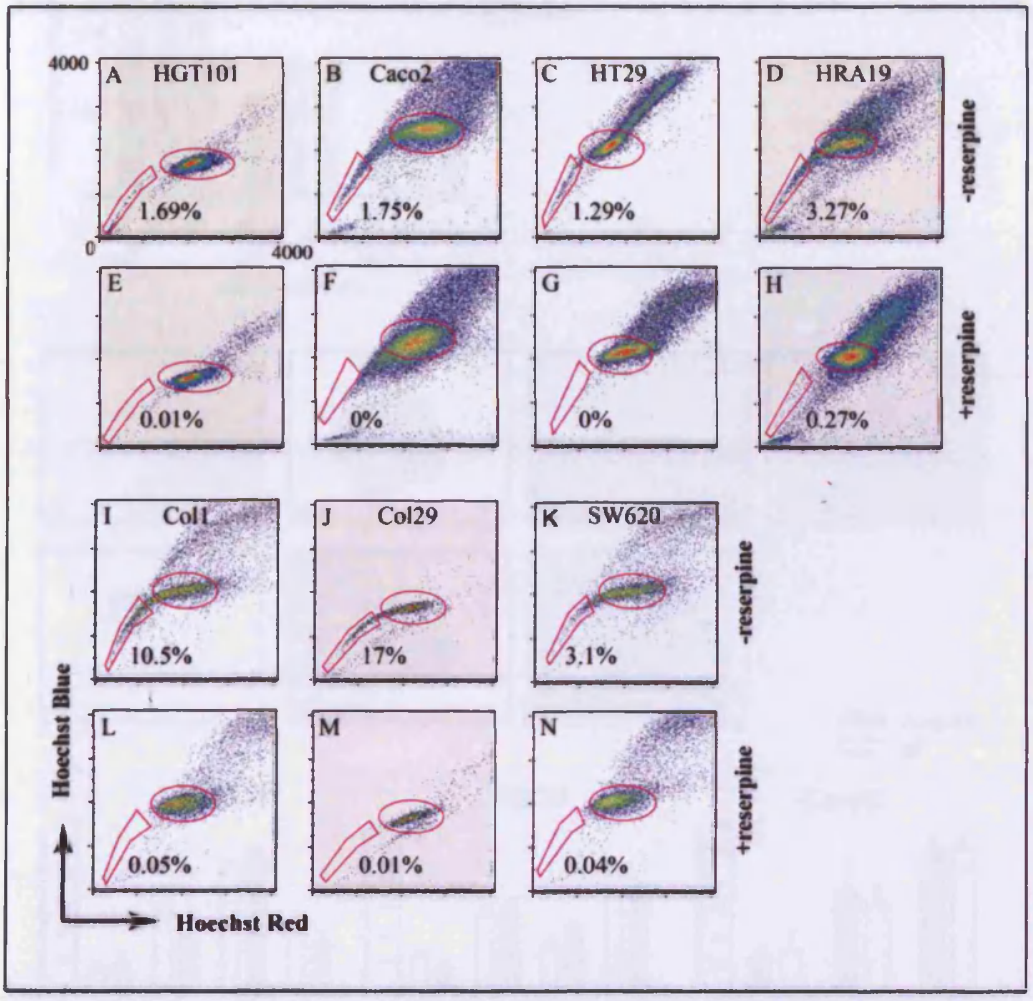


Figure 3.1. Identification of SP (lower gate) and non-SP cells (upper gate) in gastrointestinal cancer cell lines (A-D, I-K). Cell suspensions were incubated with Hoechst 33342 and analysed by flow cytometry measuring Hoechst blue vs Hoechst red fluorescence (E-H, L-N). Reserpine sensitivity was used to verify SP cells and to determine optimal staining conditions.

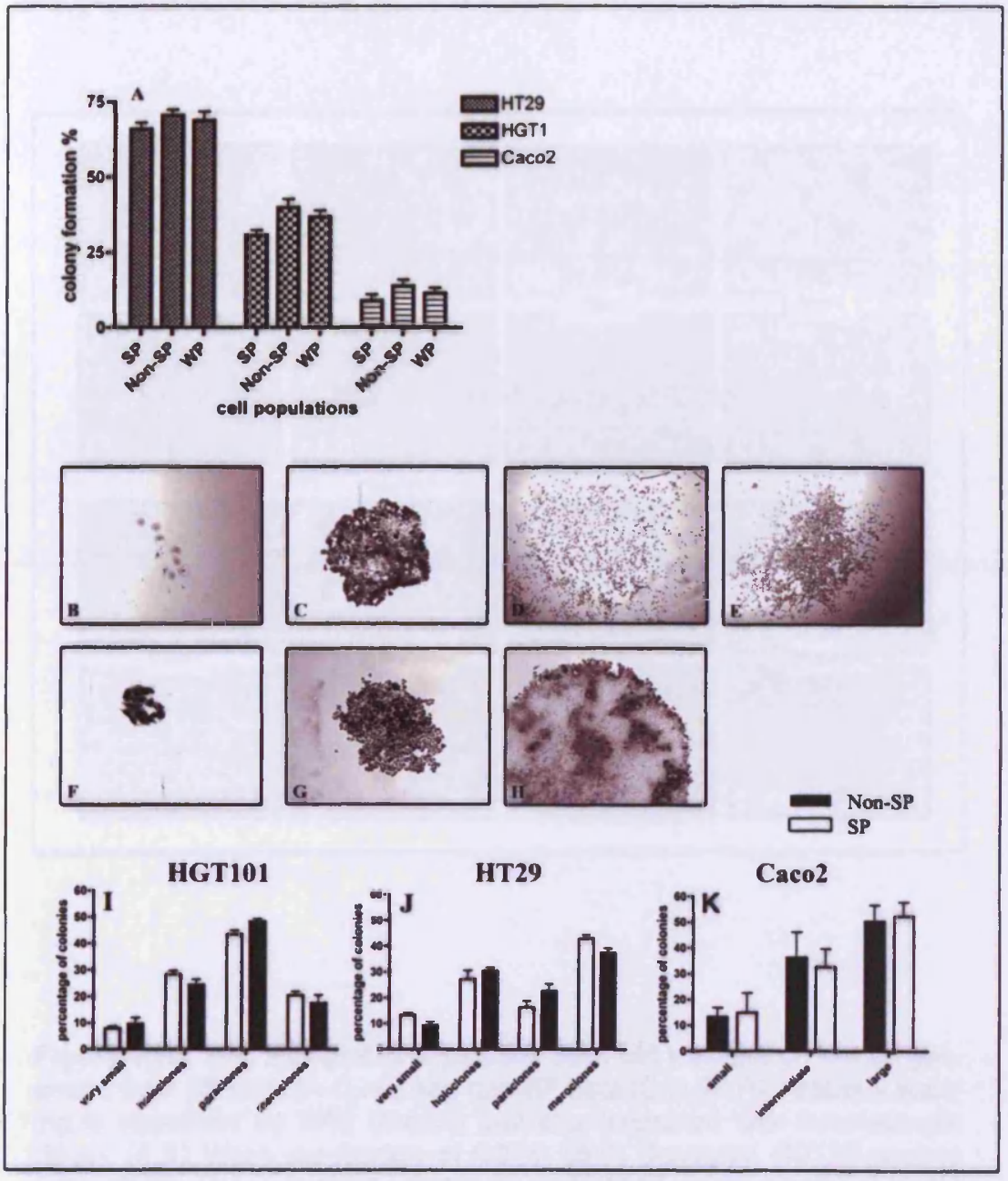


Figure 3.2a. (A) Clonogenic behaviour of SP, non-SP and whole population (WP) fractions of HT29, HGT101 and Caco2 cell lines, expressed as percentage of sorted single cells giving rise to clones after 3-week culture. (B-H) HT29, HGT101 and Caco2 clone morphologies as seen by phase-contrast microscopy (magnification x40). HT29 (shown here) and HGT101 colonies are categorised as (B) Very small (<50 cells); (C) Holoclones; (D) Meroclones; (E) Paraclones. (F-H) Caco2 colonies were categorised as (F) small (<50 cells); (G) Intermediate. (I-K) Statistical comparison of colony morphologies did not reveal a statistically significant difference between the SP and non-SP populations.

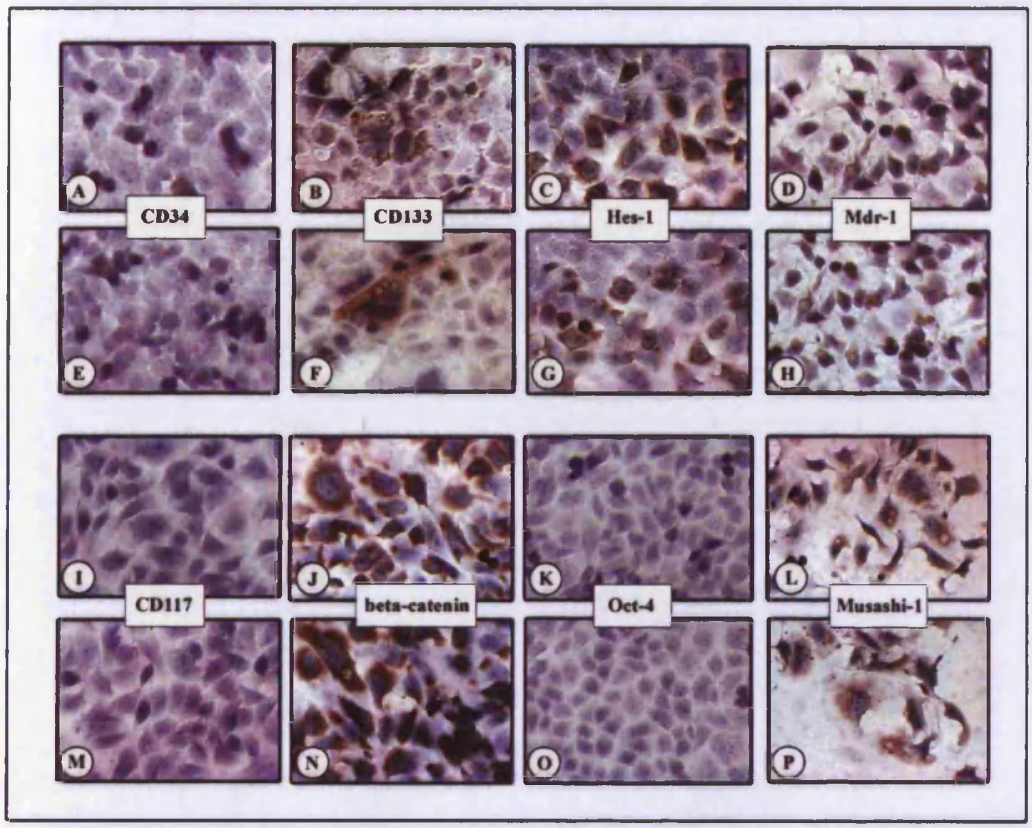


Figure 3.2b. (A) Expression of putative stem cell markers on clones generated from SP cells (A-D,I-L) and non-SP cells (E-H, M-P). Antibody staining is visualised by DAB (brown) and counterstained with haematoxylin (blue). (A,E) Weak expression of CD34. (B,F) Scattered CD133-positive cells. (C,G) Hes-1 positivity. (D,H) Sporadic Mdr1-positive cells. (I,M) No detectable CD117 expression. (J,N) Ubiquitous β-catenin positivity. (K,O) Oct-4 negativity. (L,P) Strong expression of Musashi-1.

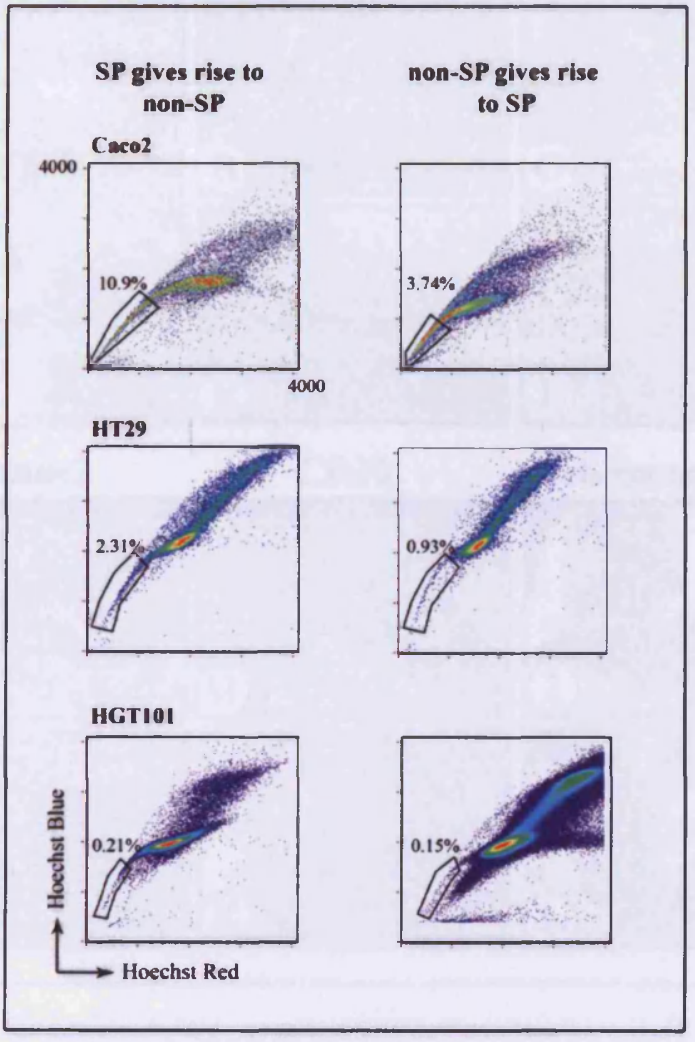


Figure 3.3. Re-analysis of sorted Caco2, HT29 and HGT101 SP and non-SP cells for the presence of the SP phenotype after 3-week culture. (A,C,E) SP cells give rise to non-SP cells; (B,D,F) Non-SP cells are capable of generating SP-cells.

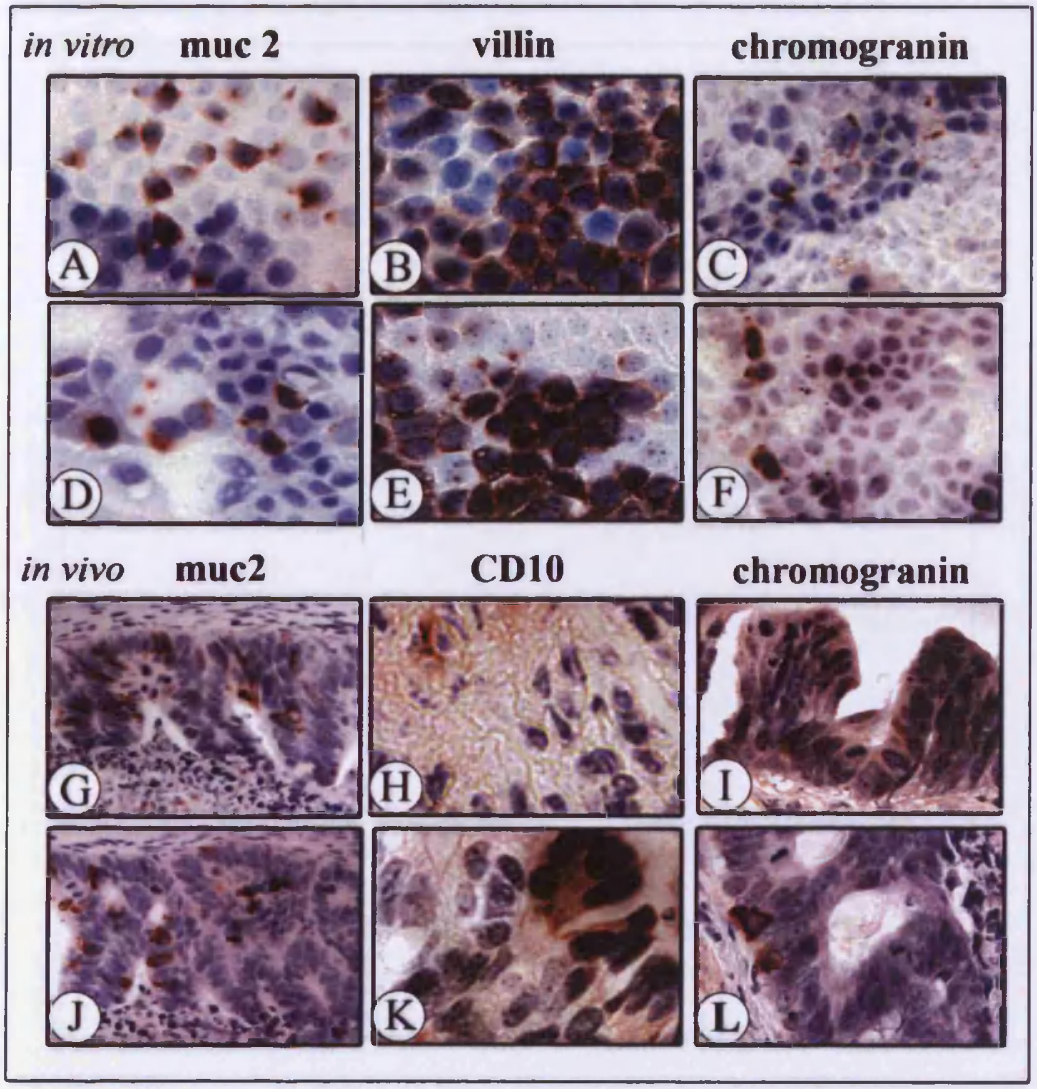


Figure 3.4. Immunohistochemistry showing potential for multilineage differentiation of SP and non-SP cells *in vitro* and *in vivo*. Antibody staining is visualised by DAB (brown), and cells are counterstained with haematoxylin (blue). (A-F) Multilineage differentiation of Caco2 SP (top row) and non-SP (bottom row) cells in vitro after 72-hour incubation with 2 mM sodium butyrate. (G-L) Multilineage differentiation of Caco2 SP (top row), and non-SP cells (bottom row) *in vivo* as seen in xenograft tumours. (A,D,G,J) Muc2 ex pression visualising mucous cells. (B,E) Strong anti-villin staining displaying enterocytic differentiation. (H,K) CD10 expression, an alternative marker for enterocytes. (C,F,I,L) Expression of chromogranin A in endocrine cells.

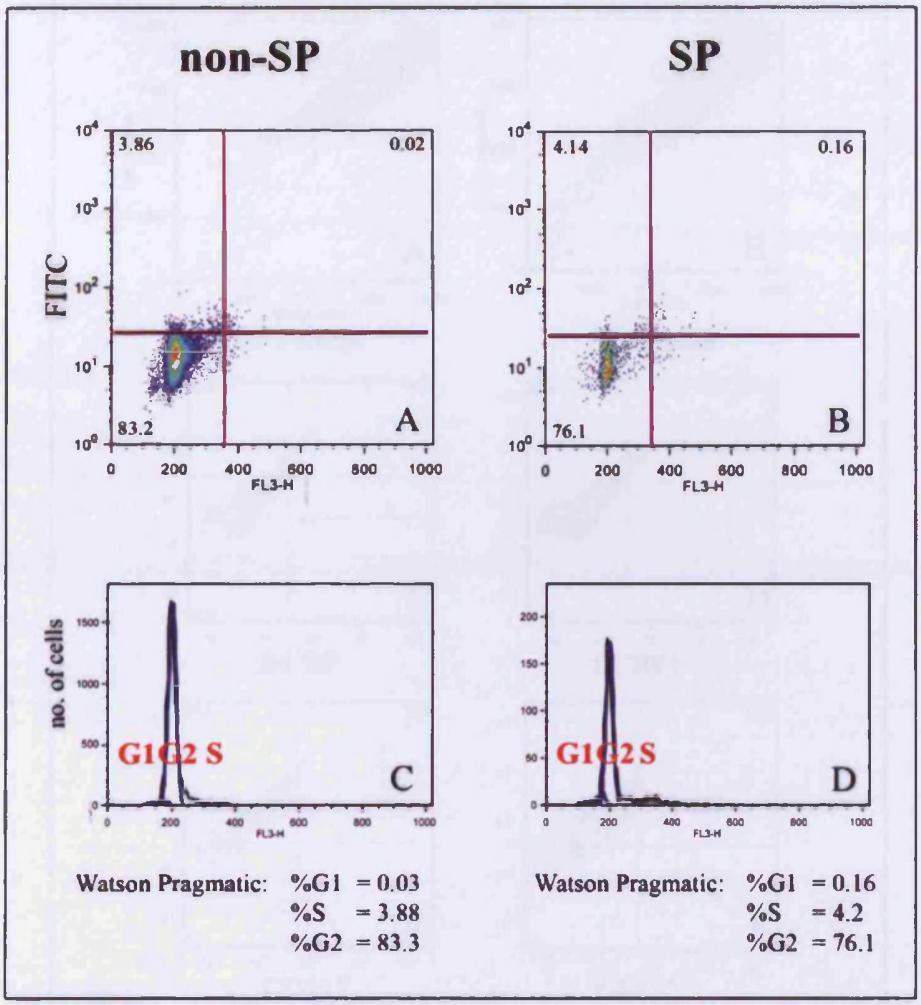


Figure 3.5. FAC analysis measuring the expression of proliferation marker Ki-67 in non-SP (left column) and SP fractions (right column). (A,B) Dot plot graphs visualising the fraction of Ki-67 expressing cells in each population. (C,D) Histogram showing fractions of cells in various cell cycle stages.

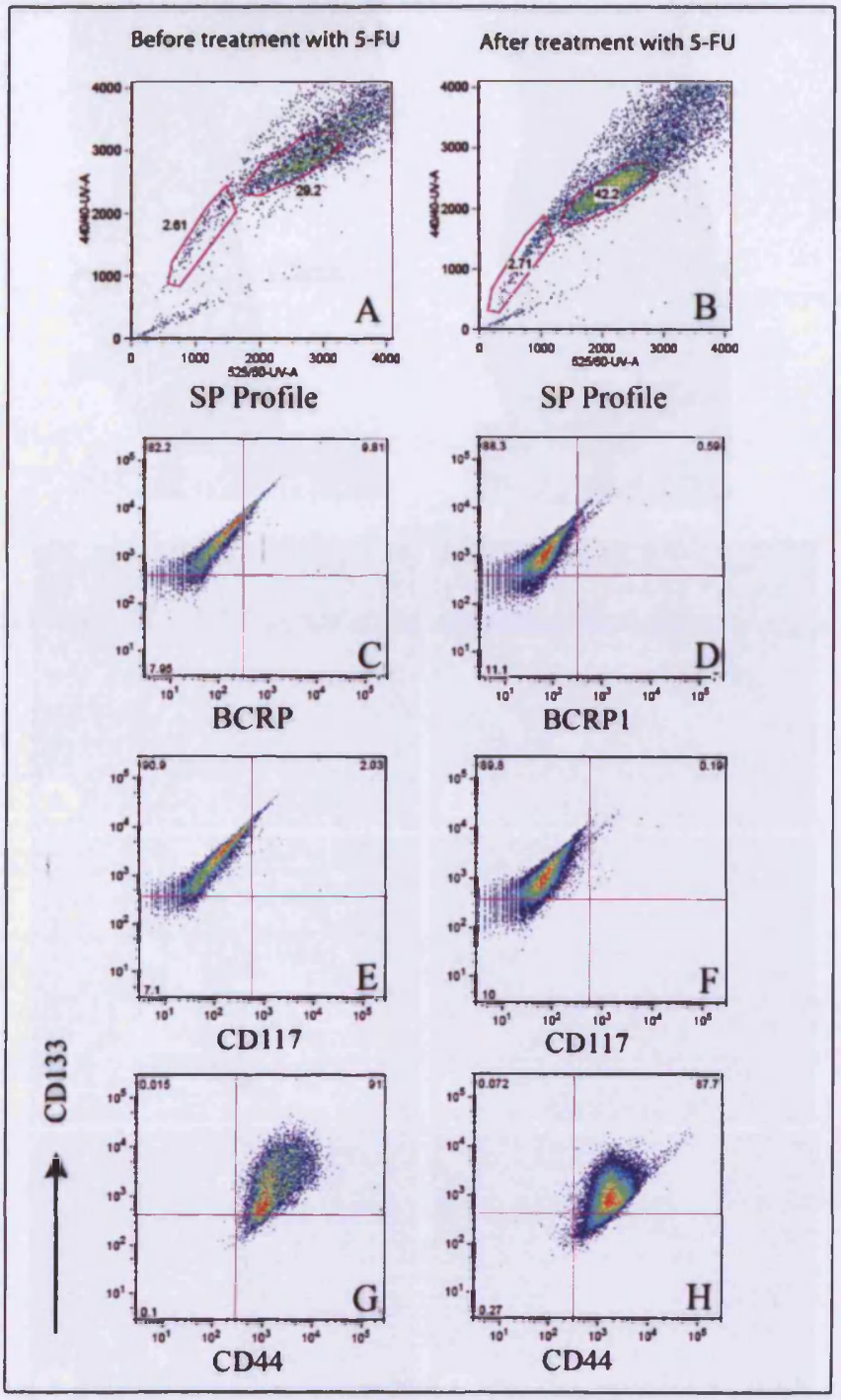


Figure 3.6. Multicolour FACS analysis showing the effects of treatment with 5-Fluorouracil (5-FU) on various colorectal cell line populations. Cell populations displayed similar fluorescent profiles before (A,C,E,G) and after (B,D,F,H) 5-FU treatment, including the SP profile (A,B), and populations positive for BCRP1 (C,D), CD117 (E,F), CD44 (G,H), and CD133 (C-H, horizontal axis).

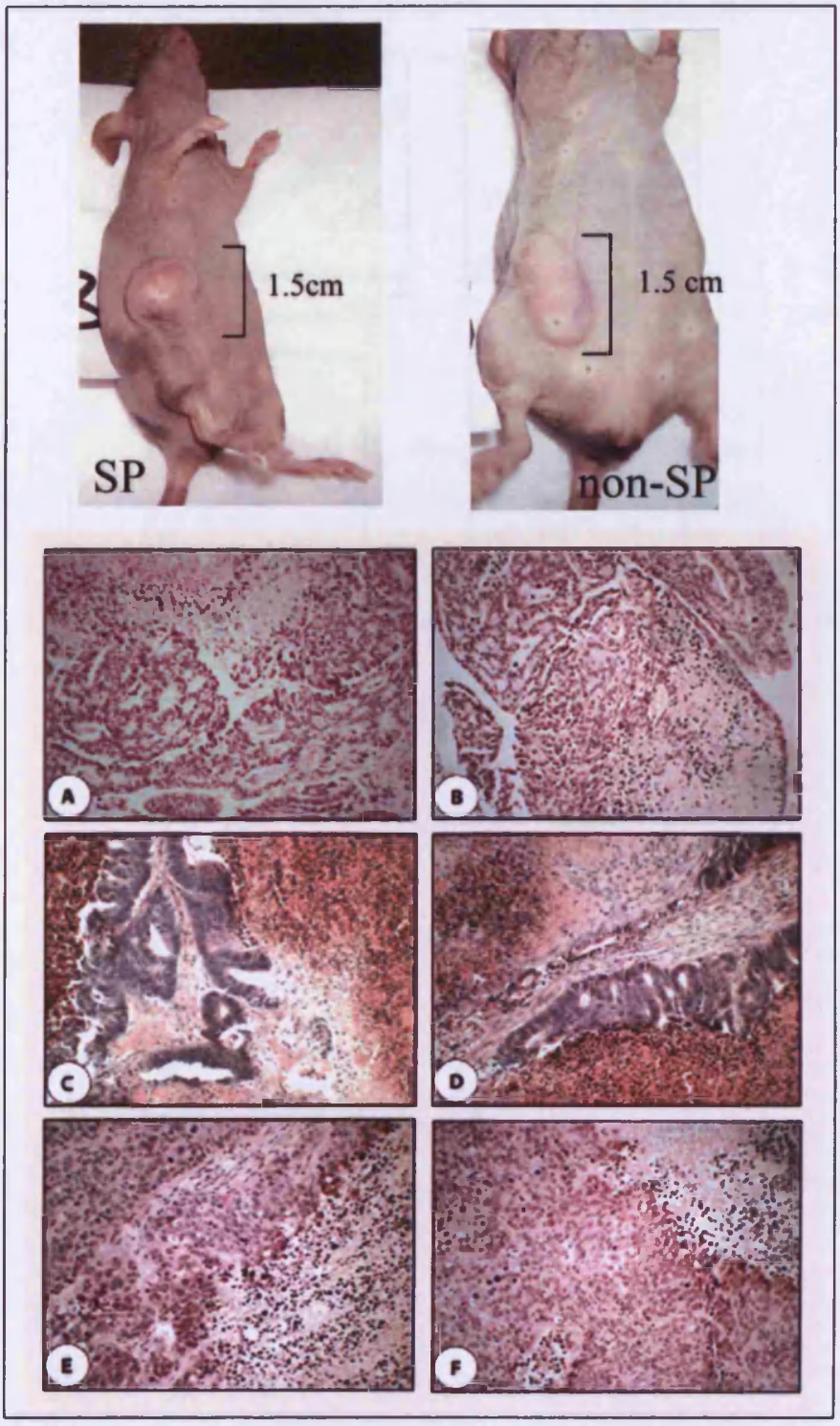


Figure 3.7. Xenograft tumours developed in nude mice originating from s.c. injection of SP (A,C,E) and non-SP cells (B,D,F). (A-F) Well-differentiated adenocarcinomas seen in Haematoxylin and Eosin stained paraffin-embedded sections of tumours originating from (A) Caco2 100 SP; (B) Caco2 100 non-SP; (C) HRA19a1.1 100 SP; (D) HRA19a1.1 100 non-SP; (E) HT29 100 SP; (F) HT29 100 non-SP cells.

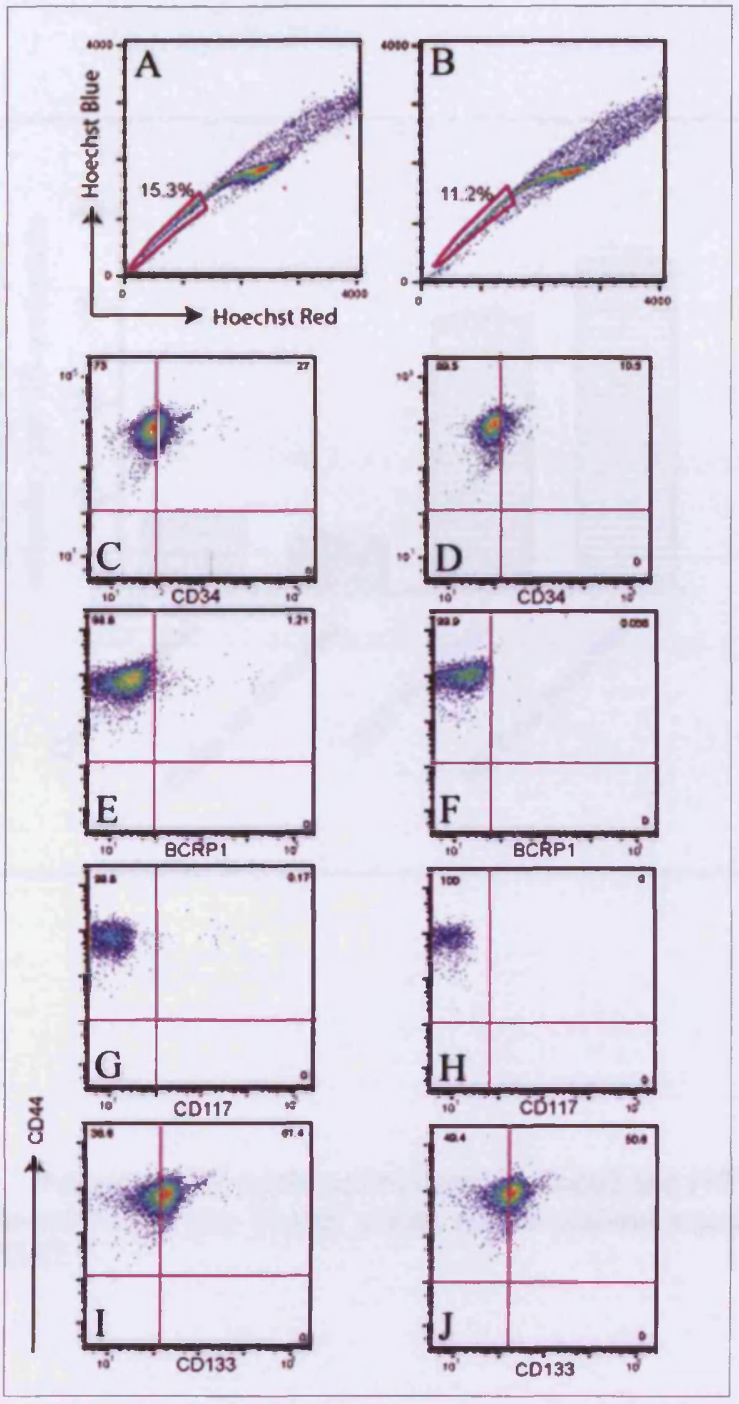


Figure 3.8. Multicolour FACS analysis of tumours generated by 100 HT29 SP cells and100 HT29 non-SP. (A,B) Reserpine-sensitive SPs appear in tumours originating from non-SP (right column) and SP (left column) cells; (C-J) Surface marker expression of tumours generated from SP (left column) and non-SP cells (right column). (C,D) Expression of CD34; (E,F) Positivity for BCRP1; (G,H) No expression of CD117; (I,J) Expression of CD133. Strong CD44 expression on all tumours is displayed on all y-axes.

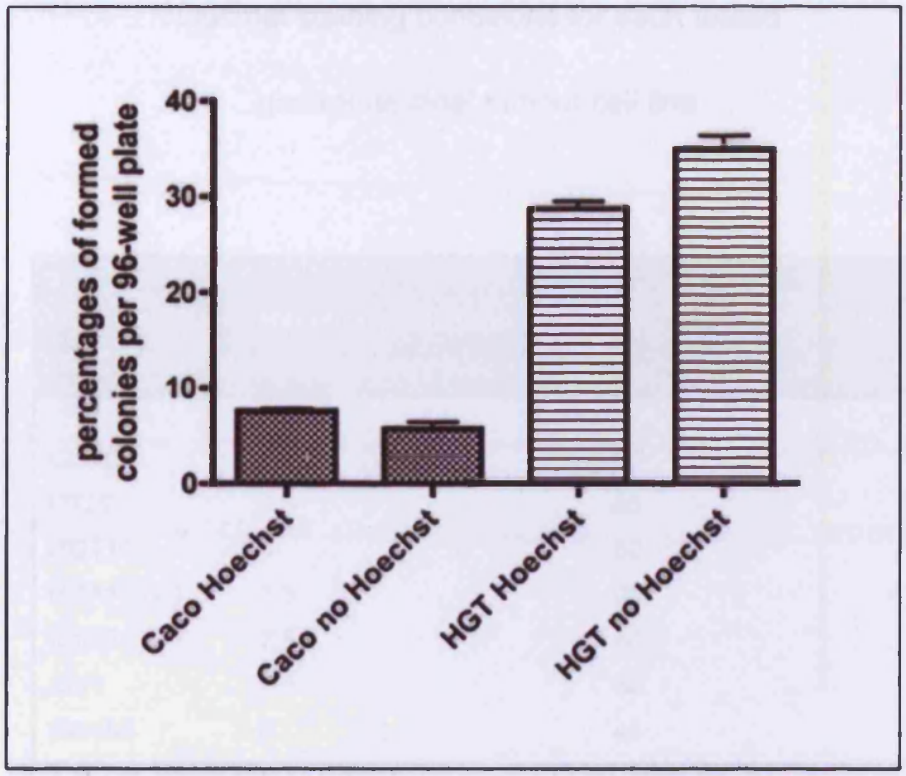


Figure 3.9. Percentage of viable colony-forming Caco2 and HGT101 cells when single-cell sorted into 96-well plates with or without incubation with Hoechst 33342.

Table 3.1

Optimal staining conditions for each tested gastrointestinal tumour cell line

Cell line	Hoechst 33342 (μg/ml)	Incubation time (min)
Caco2	6	60
HT29	7.5	60
HGT101	5	60
HRA19b1.1	7.5	90
Col29	7.5	60
Col1	7.5	60
SW480	9	45

Table 3.2

Expression of putative stem cell surface markers before and after xenografting of sorted cell fractions as determined by multicolour fluorescence analysis

Cell Line	BEFORE xenografting				AFTER xenografting		
	Marker	SP	non-SP	significant difference ($p<0.05$)	SP	non-SP	significant difference ($p<0.05$)
Caco2	CD44	87.5 ± 4.8	91.0 ± 2.9	No	94.8 ± 4.7	94.0 ± 3.2	No
	CD133	96.4 ± 3.2	92.0 ± 0.4	No	91.3 ± 7.2	92.0 ± 0.9	No
	CD117	0.03 ± 0.0	0.02 ± 0.0	No	0.8 ± 0.7	1.5 ± 0.6	No
	BCRP	1.6 ± 0.3	2.1 ± 0.1	No	4.8 ± 1.9	6.4 ± 3.0	No
	CD34	0.01 ± 0.0	2.5 ± 0.1	Yes	47.0 ± 3.7	52.1 ± 3.2	No
HT29	CD44	100.0 ± 0.0	99.9 ± 0.1	No	100.0 ± 0.0	100.0 ± 0.0	No
	CD133	93.9 ± 3.8	98.3 ± 2.3	No	61.8 ± 3.9	55.3 ± 4.2	No
	CD117	1.2 ± 0.3	1.0 ± 0.2	No	0.1 ± 0.1	0.2 ± 0.1	No
	BCRP	3.8 ± 0.8	4.3 ± 0.6	No	1.1 ± 0.2	0.8 ± 0.6	No
	CD34	0.1 ± 0.0	0.6 ± 0.1	Yes	22.6 ± 3.9	17.4 ± 6.9	No
HGT101	CD44	99.9 ± 0.1	99.9 ± 0.0	No	N/A		
	CD133	76.9 ± 2.6	82.9 ± 5.7	No			
	CD117	3.0 ± 0.8	5.5 ± 2.9	No			
	BCRP	3.7 ± 0.5	4.1 ± 1.6	No			
	CD34	30.6 ± 2.7	53.5 ± 5.4	Yes			

Table 3.3

Tumorigenicity of sorted SP and non-SP cells upon s.c. injection into nude mice

	Cell numbers injected				
	10^2	$1x10^3$	$1x10^4$	$5x10^4$	$1.5x10^5$
HT29					
SP cells	3/5	3/3	1/1	--	--
Non-SP cells	2/5	3/3	1/1	0/1	1/1
Caco2					
SPcells	1/5	1/3	1/1	--	--
Non-SP cells	3/5	2/3	1/1	1/1	0/1
HRA19					
SP	2/5	0/3	0/1	--	--
Non-SP	3/5	0/3	0/1	1/1	0/1
HGT101					
SP	0/5	0/3	0/1	--	--
Non-SP	0/5	0/3	0/1	0/1	0/1

Mice harvested after 16 weeks or when tumour burden reached a threshold diameter of 15 mm.

CHAPTER IV

CHARACTERISATION OF CELLS EXPRESSING THE PUTATIVE STEM CELL MARKERS CD44 AND CD133 FROM HUMAN ADENOCARCINOMA AND NORMAL EPITHELIUM OF THE COLON

4.1 INTRODUCTION

To date, it has not been possible to isolate and characterise the stem cells residing in the normal or diseased gastrointestinal epithelium. Partly due to the fact that there is no culture method robust enough to allow primary culture of the intestinal epithelium to test proliferative and differentiation potential of intestinal cellular subtypes, there is an ongoing debate regarding the exact number and location of intestinal stem cells. Several proteins have been implied as potential stem cell markers in the normal and neoplastic intestinal epithelium.

Most gastrointestinal cell lines contain a small SP fraction (Haraguchi *et al.* 2006). However, we have recently found in gastrointestinal cancer cell lines, that the SP phenotype does not enrich for cells with stem cell characteristics and is thus not a useful stem cell marker in these malignancies (Burkert *et al.* in Press). Two surface proteins have in the literature repeatedly been suggested as promising markers for stem cell-enriched populations.

CD44, explained in detail in section 1.4.3.2, is an adhesion molecule involved in cell adhesion, lymphocyte homing and haematopoiesis. A stem cell connection is implicated by the fact that CD44 is involved in the Wnt signalling cascade, as its expression is activated by β-catenin (Wielenga *et al.* 1999). It also appears to play a role in tumour progression and the

metastatic cascade, where it is thought to confer metastatic potential through changes in adhesion to extracellular ligands. Differential expression and gradual changes in expression of CD44 isoforms can be observed between normal, neoplastic, and metastatic tissues (Heider *et al.* 1993, Kawahara *et al.* 1996, Matzku *et al.* 1989, Yamaguchi *et al.* 1998). In breast and pancreatic adenocarcinomas, prostate carcinoma cell lines, and head and neck squamous cell carcinomas, CD44-expressing cell populations display phenotypic and functional characteristics typical for stem cell-enrichment (Al-Hajj *et al.* 2003, Ponti *et al.* 2005, Li *et al.* 2007, Patrawala *et al.* 2006, 2007, Prince *et al.* 2007). During the course of our experiment, a report emerged identifying the CD44+/EpCAM^{high} population as a tumorigenic cell population in colorectal cancers when subcutaneously injected into NOD/SCID mice (Dalerba *et al.* 2007).

CD133, described in detail in section 1.4.3.1, has been claimed to be preferentially expressed on stem cells in normal and cancerous tissues. Stem cell-characteristic behaviour has been reported in CD133-expressing populations in the normal haematopoietic system (Yin *et al.* 1997), leukaemia (Vercauteren *et al.* 2001), neural cells (Uchida *et al.* 2000), normal brain and brain tumours (Singh *et al.* 2003, 2004, Hemmati *et al.* 2003, Galli *et al.* 2004, Kuhnert *et al.* 2004, Shmelkov *et al.* 2005), kidney cancer (Bussolati *et al.* 2005, Florek *et al.* 2005), normal prostate and prostate cancer (Richardson *et al.* 2004, Collins *et al.* 2006), and recently, colon cancer (O'Brien *et al.* 2007, Ricci-Vitiani *et al.* 2007). However, a controversial report has recently emerged, indicating that the CD133+ population in C6 glioma cells is not more tumorigenic or clonogenic than its negative counterpart, and thus not enriched in stem cells (Zheng *et al.* 2007).

4.2 AIMS

CD44 and CD133 have both recently been reported as potential cancer stem cell markers in human tumours. We aimed to examine and compare the expression of CD44 and CD133 in normal human colonic mucosa and adenocarcinomas and to explore the possibility of combining both surface markers to obtain a purer population of stem cells. We attempted to test sorted fractions of cells expressing the markers to be tested for stem cell-typical behaviour such as sphere-formation *in vitro* and serially transplantable xenograft formation *in vivo*.

4.3 METHODS

All methods are described in full in Chapter II.

4.3.1 Patients and Materials

For this study, five patients that underwent surgical resection for colonic adenocarcinomas were selected. Specimens were fixed in 4% PFA and embedded in paraffin or processed to obtain single cell suspensions for FAC analysis and sorting. Ethical approval was sought and obtained as per the requirements of the United Kingdom Human Tissue Act (2006), REC reference number 07/Q1604/17.

4.3.2 Fresh tissue dissociation

Tissue from normal colon and adenocarcinomas was washed repeatedly. Normal colonic mucosa was incubated in E4 medium lacking Ca2+ and Mg, supplemented with 30mM EDTA at 37°C for 15 min, after which crypts were dislodged by vigorous shaking. Tumour tissue was minced. Both, normal and tumour tissue were then incubated in a collagenase/dispase solution in a shaking waterbath at 37°C to obtain single cell suspensions. Subsequent incubation in ammonium chloride on ice for 10 min was performed to lyse red blood cells. Finally, the cell suspensions were filtered through 70μ cell strainers.

4.3.3 Multicolour fluorescence activated cell (FAC) analysis and sorting

Single cell suspensions were incubated with directly labelled fluorescent antibodies against CD45, CD44, and CD133 on ice for 20 min. Control staining was performed with each antibody individually and all-minus-one antibodies to guide appropriate drawing of gates. DAPI was used to label and electronically gate out dead cells.

The stained cells were analysed using the LSR II Flow cytometer. Data were analyzed using FloJo computer software (Treestar, San Carlos, CA). Cell Sorting was carried out on a MoFlo™ High Performance Cell Sorter (Dakocytomation, Glostrup, Denmark) with excitation and emission wavelengths as stated in chapter II.

4.3.4 *In vitro* culture

For sphere-formation experiments, sorted cell fractions were incubated in serum-free medium at 37° C in humidified atmosphere containing 8% CO_2.

4.3.5 Xenograft experiments

Appropriate numbers of sorted cells (Tables 1 and 2) were injected s.c. into flanks of nude mice in a 1:2 mixture of PBS and matrigel. Fractions expressing the surface marker to be tested and the control population devoid of antigen expression were injected into opposite flanks.

4.3.6 *In situ* hybridisation

Sections were dewaxed, permeabilised in 1 M sodium thiocyanate, and digested in 0.4% pepsin at 37°C for 15 min. They were then fixed in PFA and dehydrated through graded alcohols. A mixture of mouse and human pan-centromeric fluorescent probes, directly conjugated to Cy-3 and FITC respectively was applied, sections were hybridised at 60°C, incubated overnight at 37°C and mounted in medium containing DAPI.

4.4 RESULTS

4.4.1 Increased expression of CD133 in colon adenocarcinomas

Expression of CD133 was compared between colon adenocarcinomas and adjacent normal colonic tissue of the same patients by immunohistochemistry. In normal colonic epithelium, the expression of CD133 was extremely sporadic, and restricted to the bottom third of colonic crypts (Figure 4.1, pg.164). In adenocarcinomas however, the expression of CD133 was highly increased. CD133 was primarily expressed on the apical surface of the cells facing the lumen of the duct-like structures that are typical in colonic adenocarcinomas. Statistical comparisons based on the immunohistochemical results were not possible due to the extreme rarity of CD133-expressing cells in normal colonic epithelium.

Double-fluorescent immunohistochemical staining for CD133 and CD44 showed CD44 on the cell-cell membranes, whereas CD133 was again restricted to the apical surface of the epithelial cells comprising the tumour. CD44 generally displayed a higher expression than CD133 and was more ubiquitously expressed throughout the entire tumours. Double expression of CD133 and CD44 was rarely seen and only on mutually exclusive sides of the cell membrane (Figure 4.2, pg.165). Due to the rare occurrence of CD133 on normal colonic tissue, we could not detect any double expression of CD44 and CD133 by fluorescent immunohistochemistry on normal colonic mucosa.

4.4.2 FAC analysis and sorting

Multicolour FAC analysis and cell sorting was performed on single cells derived from normal colonic mucosa and adenocarcinomas. Generally, both surface proteins were significantly more expressed on the cells from cancer tissues than from the normal colonic epithelium. Consequently, the fraction of double-positive cells, expressing both markers was also upregulated in the adenocarcinoma cells as opposed to the normal epithelial cells (Figure 4.3A, pg.166). CD44 showed the largest difference in

expression when comparing normal and tumour samples with average expression percentages of 0.2% ±0.009 and 2.1% ±0.470, respectively. However, when looking at the groups of tumour and normal specimens separately, CD133 showed a larger range of expression within those groups (Figure 4.3B). One of the adenocarcinomas and its adjacent normal colonic mucosa proved to be entirely devoid of CD133 expression.

The possibility of contaminating leukocytes in these experiments was ruled out by gating out CD45-expressing cells.

4.4.3 *In vitro* cultures

Cells from normal and cancer samples were sorted for single expression of CD133 and CD44, double expression of both markers, as well as the negative counterparts of each fraction. Attempts to establish *in vitro* cultures from the sorted cells in serum-free medium did not result in any surviving epithelial cells after 1 week of culture. Initial cell viability was tested before cell culture incubation to be 89%. Attempts to establish cell cultures were undertaken 5x with an identical outcome. This negative finding was not attributable to fungal or bacterial infection.

4.4.4 Lack of xenograft formation

Sorted cell fractions derived from 3 different adenocarcinomas expressing each marker separately, as well as doubly expressing cells did not result in xenograft formation up to the maximum injected cell dose of 1000 sorted cells. Injection of the negative counterparts also did not show any xenograft tumour growth. Tumour growth was monitored weekly for the entire length of the experiment of 4 months.

Injections of sorted cells derived from normal colonic epithelium were also injected in matrigel to monitor possible stem cell function resulting in crypt formation *in vivo*. H&E staining of serial sections of the injected matrigel

pellets did not reveal any crypt formation, or formation of any other recognisable structures or clusters of cells.

4.4.5 Survival of human-derived cells *in vivo*

Matrigel pellets that contained the various injected cell fractions were examined for surviving human-derived cells 4 months post-injection by mouse-human pan-centromeric *in situ* hybridisation. We could not detect human-derived cells in any of the matrigel pellets that had received cell fractions from normal colonic mucosa, as well as surface-marker negative control populations from adenocarcinomas. However, matrigel pellets that originally contained 1000 CD44+ adenocarcinoma cells showed single dispersed human-derived cells 4 months post-injection, albeit at very low numbers. Where 1000 CD133+ adenocarcinoma cells had been injected, we could detect small clusters of 2-11 surviving human-derived cells in the matrigel pellets. The most human-derived cells (66) were detected in one sample that had received 1000 double-positive CD44/133 adenocarcinoma cells.

4.5 DISCUSSION

4.5.1 Differences in surface marker expression between normal and tumour tissue

In this study, we showed that expression of putative stem cell surface markers CD133 and CD44 vary between tumour tissue of colonic adenocarcinomas and the adjacent normal colonic epithelium. Consistent with the view that cancers might possess an elevated number of stem cell-like cells, an upregulation of both surface markers appeared in the tumours vs. the normal tissues, whereby CD44 showed the largest difference between the normal and tumour counterparts. FAC analysis revealed the exact percentages of cells expressing CD133 and CD44, the location of which was visualised by immunohistochemistry. Cells expressing CD44 were widely distributed throughout the tumour masses, suggesting that its

sole expression is not indicative of stem cell status. CD133 expression was more specific, restricted to the apical surface of cells lining the duct-like structures in the adenocarcinomas. However, it has to be noted that its expression appeared uniform throughout the entire luminal epithelial sheet, or at least big stretches of it with all neighbouring cells expressing the marker, which also is not indicative of stem cells. When both surface markers were combined, only a few cells were detected expressing both markers simultaneously, on different sides of the cell membrane. These cells were sporadically distributed throughout the tumours.

4.5.2 Lack of sphere formation *in vitro*

Despite recent reports in the literature that CD133+ cells are capable of sphere formation in proliferation medium for undifferentiated cells, we could not replicate these results. After 1-week culture, no viable cells were detected, even with repeated attempts of the experiment.

4.5.3 Lack of xenograft tumour formation

Injection of various sorted cell fractions expressing combinations of cell surface markers did not result in xenograft tumour formation in any instance. Viability of the cells was tested before injection, and thus, it is conceivable that the injected cells did not represent tumorigenic populations. This indicates that neither CD44 nor CD133 are suitable candidate markers to isolate and purify cancer stem cells. However, our results stand in contrast to several reports in the literature, showing xenograft tumorigenicity of CD44+ and CD133+ cells (Al-Hajj *et al.* 2003,Singh *et al.* 2003, Hemmati *et al.* 2003, Galli *et al.* 2004, Kuhnert *et al.* 2004, Richardson *et al.* 2004, Singh *et al.* 2004, Ponti *et al.* 2005, Collins *et al.* 2006, Patrawala *et al.* 2006, Li *et al.* 2007, O'Brien *et al.* 2007, Ricci-Vitiani *et al.* 2007, Patrawala *et al.* 2007).

In retrospect, the failure to establish xenograft tumours in our experiments could be due to several reasons, mostly reflecting differences in technique.

The choice of xenograft host in the literature varies between immunosuppressed NOD/SCID mice and the slightly more immunocompetent nude mice. Although xenografts have been established in nude mice on many occasions including our own experiments with cultured cancer cells (chapter III), for several experiments with primary colonic tissue, the mouse of choice was the NOD/SCID variant. It is conceivable that the host immune response of nude mice might be too strong for the sensitive primary adenocarcinoma cells, causing their failure to produce tumours. Another difference in technique was the route of injection. In our experiments, we injected the sorted cells s.c., as this was a well-known technique for xenograft tumour formation. However, recent successful xenografts with primary colonic tissue formed upon injection of the cells into the renal capsule, where the injected cells are less likely to be dispersed throughout the body post injection (O'Brien *et al.* 2007).

One of our hypotheses was that identification of a true stem cell enriched population from normal colonic tissue would result in crypt formation and differentiation into all epithelial cell lineages of the colon *in vivo*. We based our experiments on findings by Del Buono and colleagues in our laboratory, who showed that the grafting of single crypts embedded in collagen pellets s.c. into nude mice resulted in maintenance of crypt morphology and the *de novo* formation of crypts and villi (Del Buono *et al.* 2005). It appeared as though the embedded crypts were capable of recruiting host-derived stromal cells, which provided the right microenvironment for the cryptal stem cells to proliferate. However, in our experiments, fractions of single cells did not appear to result in the recruitment of host stromal cells and in matrigel pellets that contained cell fractions from normal colonic mucosa, no surviving human cells were detected 4 months post injection. Two conclusions could be drawn from these findings: (1) CD44 and CD133 may not be useful as stem cell markers in the human colonic epithelium, or (2) Our experimental set-up did not provide the appropriate microenvironment for stem cell behaviour, rendering the results inconclusive.

4.5.4 Human-derived cells four months post-injection

Matrigel pellets extracted 4 months after injection of cells showed varying numbers of surviving human cells through detection by mouse-human pan-centromeric FISH. Interestingly, the amounts of human-derived cells in the pellets correlated to the reported stem-cell enrichment of the different cell fractions. CD44 was arguably the weakest candidate stem cell marker, and consequently, we detected only sporadic single human-derived cells in the pellets from CD44-expressing adenocarcinoma cells. Matrigel pellets from CD133+ cells showed small clusters of human-derived cells, which is consistent with claims in the literature that CD133 represents an enriched stem cell population. In one pellet that had received double-positive cells however, we observed a much larger cluster of human cells (Figure 4.4). The normal survival of epithelial cells in the human colon is shorter than the 4 months after which these cells were detected in the murine host. Consequently, it is likely that the human cells are derived from the originally injected cells, rather than representing surviving injected cells. Although this result could indicate that the CD133/44 double-positive cell fractions are able to form the largest clones, which is most consistent with stem-cell typical behaviour, such conclusions can only be drawn if the experiment was expanded to larger numbers of representative samples.

4.6 CONCLUSION

In this study, we showed that epithelial cells from adenocarcinomas of the human colon show a higher expression of putative stem cell markers CD44 and CD133. In addition, the location and expression characteristics of both surface markers was visualised within the adenocarcinoma sections. By combining the two cell surface markers, we sought to find a population of cells more highly enriched in cells with stem-cell characteristics than the reported enrichment of each population separately. Stem cell-typical behaviour such as self-renewal and multipotential differentiation has been experimentally demonstrated by sphere formation *in vitro* and serial transplantable formation of xenograft tumours. In our experiments, we could not observe sphere formation or the ability to generate xenograft

tumours in any of the cell fractions tested, which might reflect the absence of stem cell-like cells in the CD44, CD133 and CD133/44 populations, but could also be due to differences in technique. However, human-derived cells were detected 4 months post-injection in matrigel pellets from CD44, CD133, CD133/44 fractions in quantities reflecting the presumed stem cell enrichment of these fractions. Under-representative numbers and lack of stem cell characteristics do not allow for conclusions to be drawn from these findings.

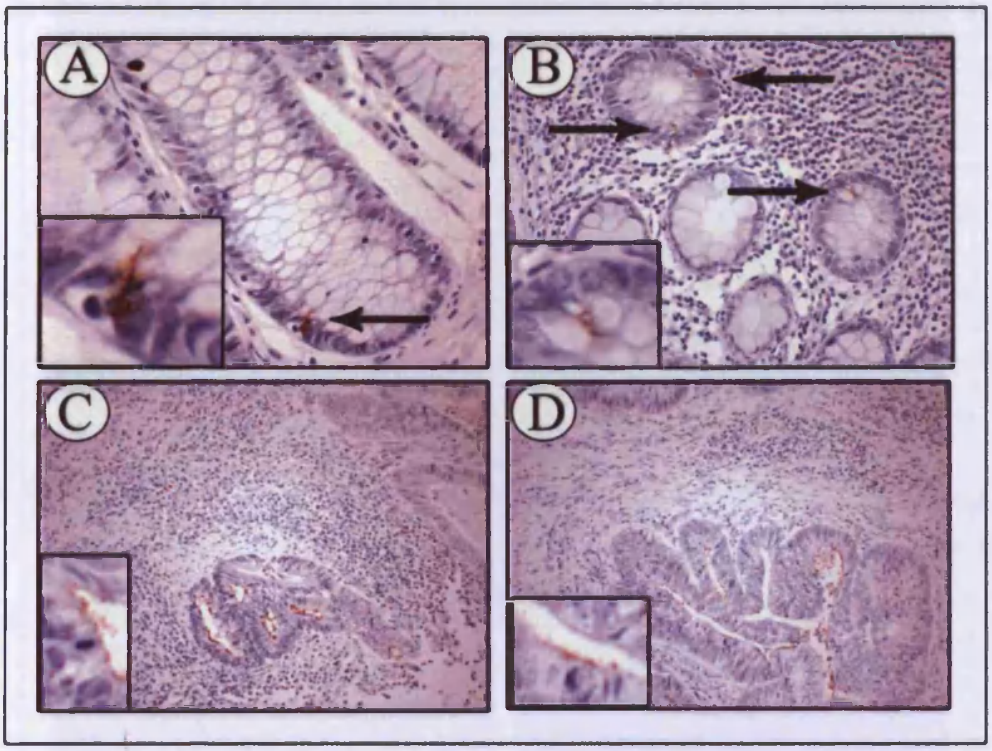


Figure 4.1. Immunohistochemistry to show expression of CD133 on (A,B) normal colonic epithelium and (C,D) colonic adenocarcinoma. Sporadic CD133-expressing cells are detected in normal colonic sections (arrows), whereas its expression is upregulated in adenocarcinoma of the colon, mostly seen on the apical surface of gland-like structures.

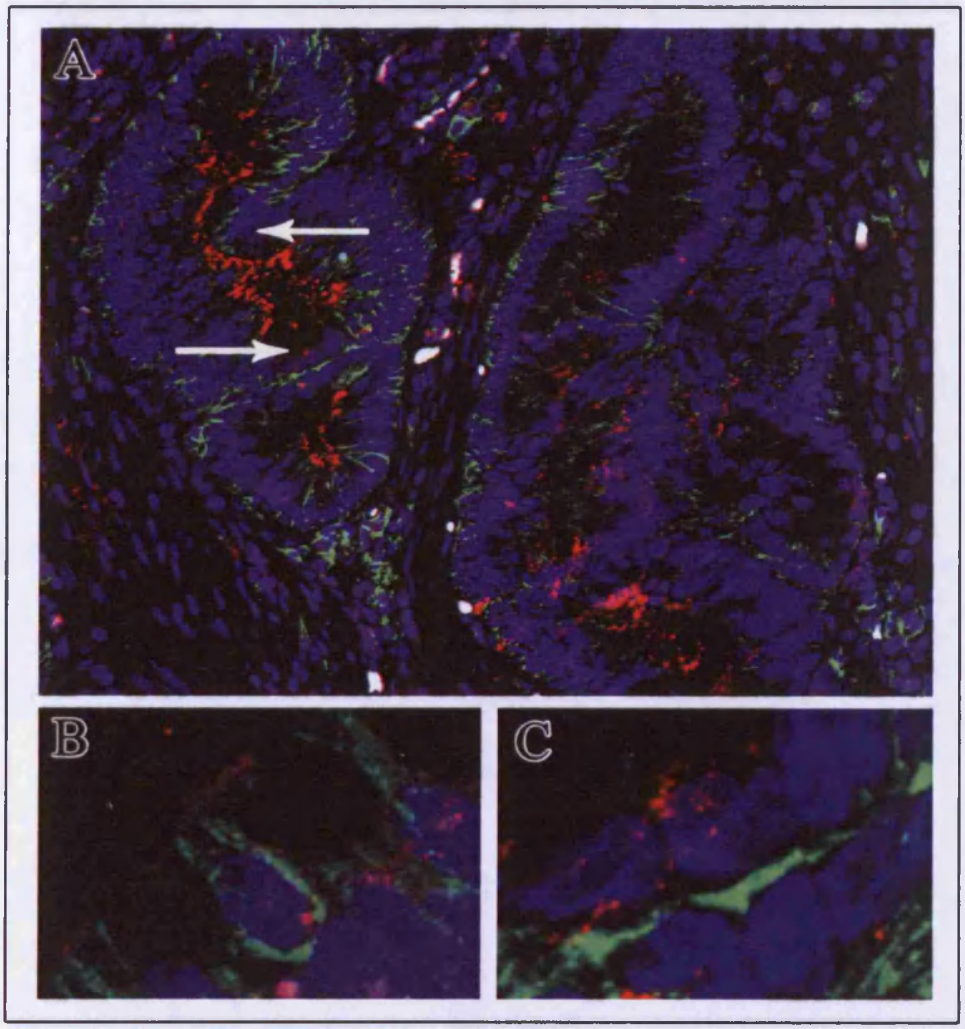


Figure 4.2. Expression of CD44 (green) and CD133 (red) shown by fluorescent double immunohistochemistry on a human colonic adenocarcinoma. CD133 expression is mainly seen on the apical surface, whereas CD44 is expressed on the entire cell membrane. (B,C) Higer magnification images of cells expressing both surface markers.

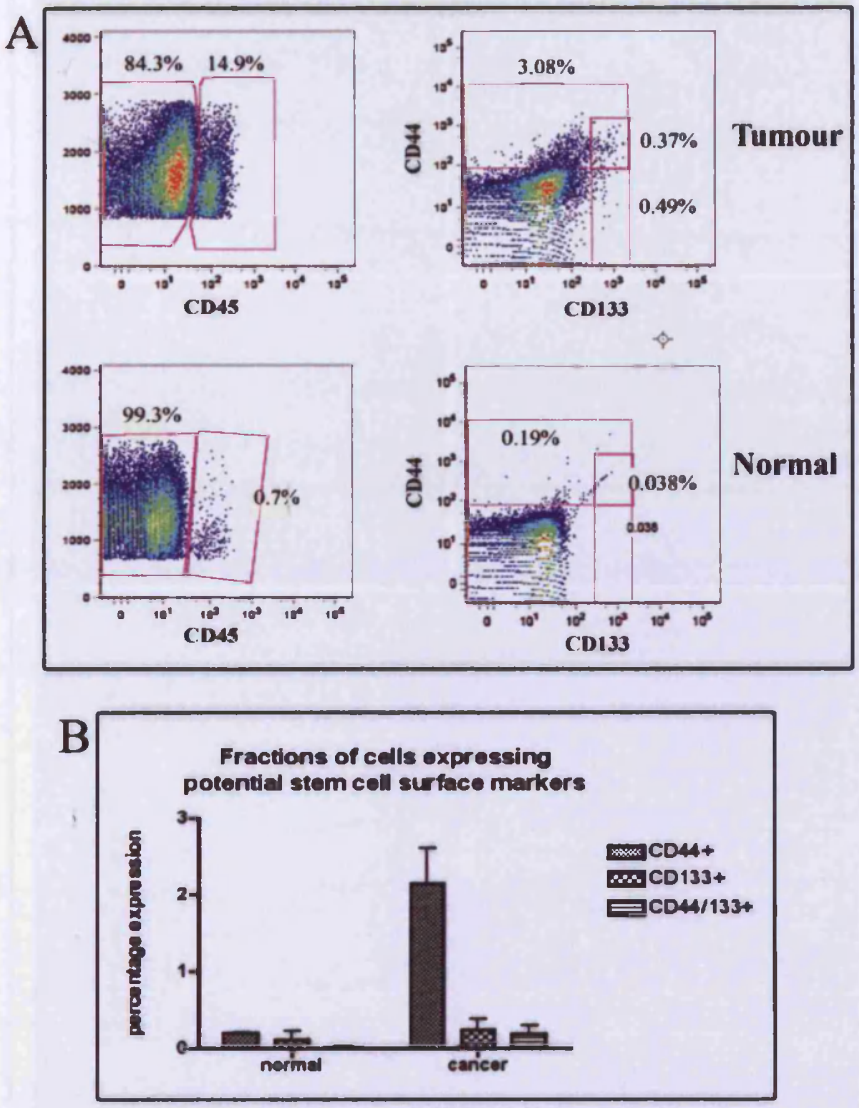


Figure 4.3. Expression of surface markers on single cell suspensions derived from colonic adenocarcinomas and adjacent normal tissue. (A) Multicolour FAC-analysis showing the expression of CD45 on the dissociated cells and the expression of CD45 and CD133 on the gated CD45-negative cell population. (B) Statistical analysis of surface marker expression on normal and cancer epithelial cells, tested in triplicates.

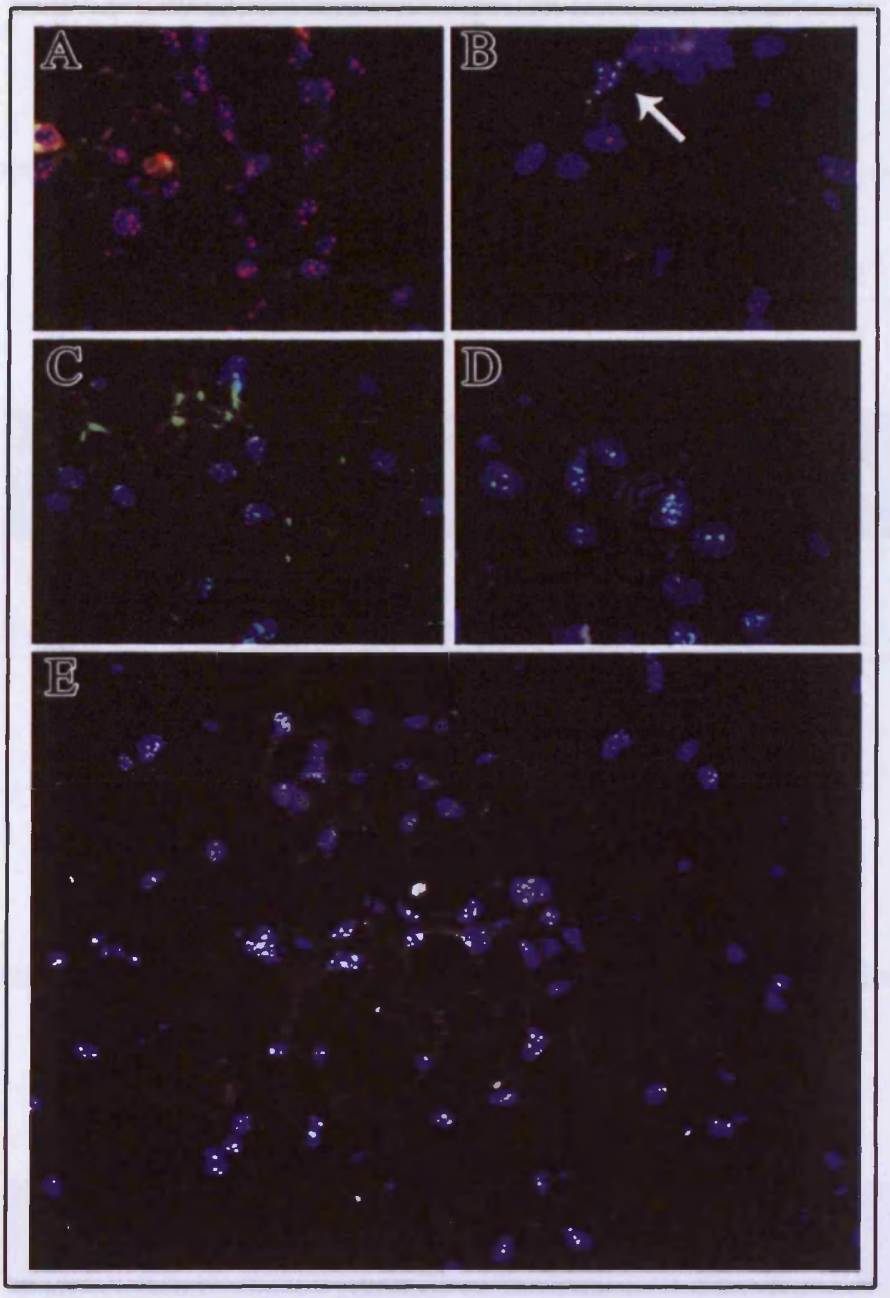


Figure 4.4. FISH with a mixture of mouse-human pan-centromeric probes to visualise cells derived from the human injected cell populations (green) and mouse host cells (red) within matrigel pellets. (A) Injection with sorted cells from normal colonic epithelium did not show any surviving human cells. (B) Single human cells in mice injected with tumour-derived CD44+ cells. (C, D) Small clusters of human cells in mice injected with CD133+ cells. (E) Larger cluster of human cells derived from CD133/44 double positive cells.

Table 4.1

Tumour development in mice injected with sorted cell populations derived from adenocarcinomas of the colon.

Number of injected cells	CD133		CD44		CD133/44	
	+	-	+	-	+	-
100	0/6	0/6	0/6	0/6	0/6	0/6
1000	0/6	0/6	0/6	0/6	0/6	0/6

Table 4.2

Crypt development in mice injected with sorted cell populations derived from normal colonic epithelium

Number of injected cells	CD133		CD44		CD133/44	
	+	-	+	-	+	-
100	0/6	0/6	0/6	0/6	0/6	0/6
1000	0/6	0/6	0/6	0/6	0/6	0/6

CHAPTER V

DONOR-DERIVED CELLS IN SKIN TUMOURS AFTER RENAL TRANSPLANTATION

5.1 INTRODUCTION

The capability of bone marrow (BM)-derived cells to engraft into multiple organs is well documented and has in recent years attracted substantial attention from the research community. Plasticity of transplanted BM cells has been shown in mouse models, as well as in humans, and has been observed in many tissues. The extent of BM engraftment varies in different epithelial tissues. In general, it reflects the rate of turnover of the tissue in question and is enhanced by injury, suggesting that haematopoietic stem cell-derived inflammatory cells contribute to epithelial repair.

5.1.1 Engraftment of transplanted cells into the skin

In the normal murine skin, the engraftment of donor-derived cells is sporadic and amounts to 1-14% of the total cell population (Krause *et al.* 2001, Badiavas *et al.* 2003, Kataoka *et al.* 2003, Harris *et al.* 2004, Brittan *et al.* 2005). The contribution of BM cells to dermal fibroblasts is larger, reaching up to 15-20% of the total fibroblast poulation (Fathke *et al.* 2004). In the human skin, engraftment of BM cells into the normal epidermis is rare and reports vary between complete absence (Cogle *et al.* 2007, Murata *et al.* 2007), and 0.07% (Avital *et al.* 2007), 2-7% (Korbling *et al.* 2002) and even as high as 14.8% (Hemmati *et al.* 2003). It has been suggested that epidermal engraftment only occurs in patients with graft-versus-host disease (GvHD), specifically in areas of heightened tissue damage (Murata *et al.* 2007).

Importantly, in mice, but not in humans, BM-derived cells in the skin have been found in small clusters in known stem cell areas, such as the CD34-positive bulge region of the hair follicle and the epidermal proliferative unit (EPU) in the interfollicular epidermis, and thus can presumably contribute to epithelial homeostasis. They were also shown to actively proliferate by incorporation of BrDU and to be capable of colony formation *in vitro* (Brittan *et al.* 2005). Contrasting observations have been made in humans. Clusters of BM-derived epidermal cells have not been shown to date, and when Hemmati and colleagues (2003) attempted to culture keratinocytes of bone marrow recipients in conditions favourable for keratinocyte stem cells, no colonies of BM-derived cells were seen, even though the observed BM-contribution to epidermal cells reached up to 14%. This indicates that, while BM-derived cells may engraft in stem cell niches in the mouse epidermis, this phenomenon may not apply to humans.

In disease scenarios, BM engraftment appears to underlie different principles than in normal tissues. Acute injury enhances the engraftment of BM-derived cells significantly, however, in a transient manner. Fathke *et al.* (2004) noted that the majority of BM-derived cells in areas of acute tissue damage or acute inflammation express the haematopoietic stem cell marker CD34: with time, this expression decreases and most BM-derived cells express keratinocyte and fibrocyte markers. Thus, they propose a logical mechanism whereby inflammatory cells are recruited from the BM to the site of injury, where they aid in tissue regeneration by transdifferention into keratinocytes. Subsequently, the tissue follows its normal migratory pattern of tissue homeostasis and the BM-derived cells gradually diminish in number.

In areas of chronic inflammation, engraftment is not only profoundly enhanced in quantity, but donor-derived cells are seen in patterns that are typical for cells derived from a common ancestor (Houghton *et al.* 2004). This is different from normal tissues, where BM cells are mostly incorporated as sporadic cells, not as clones, suggesting that there they do not proliferate or act as stem cells. It appears as though in most cases, an

environment of continual damage such as chronic inflammation is required for BM-derived cells to engraft as stem cells into epithelia. Notably, this is different from acute tissue damage or transient infections, where BM-derived cells to not appear to engraft as stem cells. A possible explanation for this scenario may be that permanent tissue damage eventually results in failure of the resident tissue stem cells, whereupon BM cells are recruited to fill the free stem cell niche and permanently act as the new resident tissue stem cells. This paradigm can be extended to the development of epithelial cancers. Acting as tissue stem cells, the BM-derived cells have the longevity to acquire the changes required to transform into cancer stem cells.

5.1.2 Donor-derived cells in post-transplant cancers

The development of cancerous lesions is a well-known after-effect of tissue and cell transplantations, attributable to risk factors such as immunosuppression, UV-irradiation and Human papillomavirus (HPV)-infection.

Cells of donor-origin have been demonstrated to contribute to post-transplant cancers to a variable extent. In most cases, the engraftment of BM cells in tumours appears to be similar to the engraftment in normal tissues; merely as a sporadic contribution. In a recent study, Cogle *et al.* (2007) observed a 1-4% contribution of BM-derived cells to human colonic adenomas and a 20% engraftment in lung cancers of BMT recipients, similar to the cell fractions found in normal tissues. This led the group to the conclusion that epithelial engraftment into post-transplant cancers occurs in a similar fashion to normal tissues, merely as a form of cell mimicry. BM-derived cells have been found in various tumour-associated structures. Both, Duda *et al.* (2006) and Nolan *et al.* (2007) found a BM cell contribution to the neovascularisation of tumours. In our laboratory, it was shown that up to 25 % of stromal myofibroblasts in a mouse model of pancreatic insulinoma can be donor-derived (Direkze *et al.* 2004).

However, it has also been shown that post-transplant lesions can be completely composed of BM-derived cells. For example, Papadopoulos *et al.* (1994) have found a leukaemic clone entirely consisting of donor-derived cells in a BM transplant-recipient. In an important study, Houghton *et al.* (2004) proved the BM origin of entire solid tumours in a mouse model of gastric cancer after chronic inflammation. Since then, BM cells have been shown *in vitro* as a potential source of malignancy in several different tumour types, including epithelial, neural, muscular tumours, tumours of fibroblasts and tumours of endothelial origin (Liu *et al.* 2006).

Engraftment of donor cells has also been observed after solid organ transplantations. In a kidney transplant recipient, a promyelocytic leukaemic clone completely derived from the donor has been found (Bodo *et al.* 1999). Barozzi *et al.* (2003) dixcovered neoplastic donor-derived endothelial cells in Kaposi sarcomas in recipients of liver allografts. Notably, these are tumours of haematopoietic origins and the engrafted cells could be derived from haematopoietic progenitors carried over with the graft. One example exists of a basal cell carcinoma in a female renal transplant recipient of a male kidney that appears to be mostly composed of male cells. However, due to unavailability of donor-specific information, it was not possible to determine whether the male cells were derived from the grafted organ or from peripheral blood microchimerism (Aractingi *et al.* 2005).

These few observations suggest that transplanted cells may act as cancer stem cells in the host. These could either be neoplastic progenitors transmitted with the transplanted organ, or cells from the grafted organ, capable to transform into cancer stem cells within the host. Whether these cells are haematopoietic stem cells carried over with the transplanted organ, or another population of cells residing within the organ, capable of plasticity, is not clear.

5.2 AIMS

This project was undertaken to elucidate the engraftment of donor cells in solid tumours of solid organ recipients.
In a study involving 35 female recipients of sex-mismatched kidney allografts with a total of 176 skin tumours, we aimed to investigate the incorporation of donor cells within post-transplant secondary lesions in more detail.

By using various types of skin tumours we aimed to test the extent, location and potential cell type preferences of donor cell engraftment on a large scale. Additionally, we aimed to investigate whether donor-derived cells from solid organ transplants can engraft into the skin as cancer stem cells, giving rise to entire clonal post-transplant skin lesions and thus represent an additional risk and side-effect of solid organ allografts.

5.3 METHODS

All Methods are described in full in Chapter II.

5.3.1 Patients

35 female recipients of male kidney allografts that had developed skin lesions following the transplant were identified. From these patients, archival paraffin-embedded biopsies from 176 skin lesions with varying histopathological diagnoses were used for this study (Table 5.1, pg.197). Ethical approval was sought and obtained as per the requirements of the United Kingdom Human Tissue Act (2006), REC reference number 06/Q0603/1.

5.3.2 Quantitative PCR (qPCR)

Serial sections of every lesion were dewaxed and scraped off with a sterile needle into proteinase K solution (Picopure) for overnight digestion. Known amounts of the extracted DNA were subjected to qPCR using the Y-

quantifiler kit (Qiagen) with primers specific to a sequence on the sex-determining gene *SRY* on the Y chromosome. Results were analysed using the standard curve method.

5.3.3 Fluorescent *in situ* hybridisation (FISH)

Sections were de-waxed and then subjected to the FISH-protocol as described in chapter II. For XY-FISH, a probe mixture was used consisting of Cy3-labelled Y chromosome-specific probes and FITC-labelled X chromosome-specific probes. For Y-FISH, a FITC-labelled probe specific to the Y chromosome was utilised. The temperature used for hybridisation was 80°C. Probes were visualised under the fluorescent microscope.

5.3.4 Immunohistochemistry combined with Y-FISH

Sections were dewaxed, subjected to antigen retrieval as needed and stained as described in Chapter II, using primary antibodies against pan-cytokeratin, α-SMA, CD34, or HPV. Staining was performed using the alkaline phosphatase technique and visualised with Vector Red. After immunocytochemical staining, the Y-FISH protocol was followed, with adjusted digestion times depending on the method used for antigen retrieval.

5.3.5 Statistical analyses

All statistical analyses were conducted using Mann-Whitney and Kruskal-Wallis analyses with Dunn's post-test for non-parametric data and the Student's two-tailed t-test for parametric data where appropriate (Prism, GraphPad, San Diego). Binomial data was analysed using the chi-square test of statistical signficance. A probability (P) value of ≤ 0.05 was taken as an appropriate level of significance.

5.4 RESULTS

5.4.1 Post-transplant skin tumours containing Y-chromosomal genetic material

Quantitative PCR for the sex-determining gene *SRY* in the Y chromosome was sensitive enough to detect as few as 4 Y chromosome copies among 15,000 cells (equating to 50 ng DNA). Of the 176 tested skin biopsies, 18.75% contained Y-chromosomal genetic material. These were distributed amongst 46% (16/35) of the female transplant recipients. Most patients displayed multiple lesions and on average 27% of an individual's skin tumours contained male chromosomal material (Figure 5.1a, pg.186).

We detected Y chromosome-positive cells in squamous cell carcinoma (SCC), basal cell carcinoma (BCC), carcinoma *in situ* (CIS) and actinic/bowenoid keratosis (AK) samples with distinct engraftment percentages across the different lesion types (Table 5.1, Figure 5.1b). The percentage of Y-positive tumour samples ranged between 15-27%, whereby there was a higher percentage of Y-positive cells amongst BCCs (23%) and AK/BK (27%) tumours than amongst SCCs (15%) and CIS (19%). We could not find any tumours containing Y-positive cells in sections from porocarcinoma, dysplastic warts and lentigo maligna samples (Figure 5.1b,A, pg.187).

When the tumour types were grouped into malignant and premalignant categories, the percentage of Y-containing samples in the premalignant category was 20%, compared to 18% in the malignant category (Figure 5.1b,B, pg.187). Statistical analyses using the chi-square test revealed no statistically significant difference between the different tumour types or malignant and premalignant categories.

5.4.2 Quantities of Y-positive cells determined by qPCR

By qPCR, the contribution of Y-positive cells to the entire tumour was found to be on average 1.6%, ranging between 0.02%-10.68% per 50 ng of DNA tested, which equates to 4-1626 Y chromosome copies/15,000 cells. The

percentage engraftment of Y-positive cells showed a distinct pattern when the different tumour types were compared (Table 5.1, Figure 5.2A, pg.188). BCCs, for example, contained significantly higher amounts of Y-positive cells than CIS samples (P=0.032) and when the categories of malignant and premalignant tumours were compared, there was a statistically significant difference regarding the amount of Y-positive cells in the tumours (P=0.009) (Figure 5.2B, pg.188).

5.4.3 Sporadic distribution of Y-chromosome-positive cells with no evidence for cell fusion

Exact quantities of Y-chromosomal copies within the entire lesions were determined by qPCR. Since these purely quantitative data do not reveal information regarding the location and identity of the donor-derived cells, we used XY-FISH to visualise Y-positive cells. Location and morphology of the Y-positive cells allowed us to determine cell type and location preferences for engraftment. XY-FISH on male control samples of BCC and SCC tumours revealed that due to unaccessability of nuclei due to tissue sectioning and orientation, only 71% of all nuclei could be successfully labelled. No difference was seen in labelling-efficiency between different tumour compartments, such as tumour epithelium, normal epithelium and tumour stroma. We thus applied a correction factor of 1.4 to all counted cell numbers to adjust for the expected underestimation.

Of the post-transplant tumours that upon qPCR showed >1% engraftment, 5-7 samples of each tumour type were selected for further examination by XY-FISH and combined immunohistochemistry and Y-FISH. For each analysis, no less than 130 (130-300) cells were counted per sample.

XY-FISH samples showed sporadic incorporation of donor-derived cells within the tumours, irrespective of tumour-type and latency of tumour development. We reliably detected sporadic donor-derived cells in the epithelial sections as well as in stromal sections of the samples, determined by morphology (Figure 5.4, pg.190). We could not detect any clusters or

clones of donor-derived cells in the analysed sections. In every tested section, we also checked for events of cell fusion, which would be evident by an increased number of sex chromosomes in any given cell. Having evaluated more than 3000 cells, we could not find any evidence for cell fusion.

5.4.4 Engraftment of Y-positive cells into various cell types

5.4.4.1 Engraftment in CD45-expressing cells

To identify preferential location of potential infiltrating donor-derived CD45-expressing lymphocytes and to identify possible peripheral blood microchimerism in the transplant patients, immunohistochemistry for the pan-leukocyte antigen CD45 and combined CD45-immunohistochemistry and Y-FISH were performed on selected tumour sections.

CD45-positive cells were rarely detected dispersed within the tumours, but mostly surrounding the tumours and infrequently in separate small pockets within the tumours (Figure 5.5). When 1880 cells from 10 separate tumours were examined, no peripheral blood microchimerism was found as only 2 double-positive cells were detected overall.

5.4.4.2 Engraftment in epithelial cells

The epithelial nature of donor-derived cells was indicated by the large, round morphological phenotype typical for epithelial cells. By combined immunohistochemistry for pan-cytokeratin antigen expression, combined with Y-FISH, we were able to confirm that Y-positive cells can be of epithelial nature (Figure 5.6, pg.192). Epithelial Y-positive cells were seen sporadically distributed both, in the normal epithelium, and in epithelial tumour cells with AK/BK sampled harbouring a particularly low percentage of Y-positive cells. No significant difference was seen between the percentage of incorporated cells in BCCs (4.2±0.9%), SCCs (5.7±1.7%), CIS (4.0±1.7%) and normal epithelium samples (6.5±1.2%). AK/BK samples showed an average Y-positive percentage of 0.6±0.5% of all epithelial cells. This fraction differed significantly from BCCs (P=0.015),

SCCs (P=0.031) and normal epithelium (P=0.0054). Again, no clusters of Y-positive cells were observed.

5.4.4.3 Engraftment in stromal myofibroblasts

Immunohistochemistry for α-SMA combined with Y-FISH showed that male-derived cells contribute to stromal myofibroblasts in a sporadic fashion (Figure 5.7, pg.193). Percentages of Y-positive cells among α-SMA-expressing cells were found in the range of 13.5-15%. There was no significant difference between the different tumour types, and thus, the average engraftment could be determined as 14.1±1.1% The identity of the engrafted cells could also be confirmed by their elongated, flat morphology.

5.4.4.4 Engraftment in endothelial cells

Using the same technique, Y-positive cells were found in cells expressing the endothelial marker CD34. However, this was an extremely infrequent event, only seen in 2 of 300 counted cells. CD34-positive cells can be observed as blood-vessel forming tissue components, but also as single cells distributed throughout the tissues. These could represent infiltrating haematopoietic stem cells or stem cells within the skin, since CD34 has also been implicated as a putative marker for keratinocyte stem cells. In our observations, we only detected Y-positive cells within the vessel-associated CD34-cells, but never amongst the single CD34-positive cells (Figure 5.8, pg.194).

5.4.4.5 Susceptibility of engrafted cells to HPV infection

The proneness of transplanted patients to HPV-infections was confirmed by immunohistochemistry. Y chromosome-positive cells were seen amidst HPV-infected areas and the combined staining technique confirmed that the engrafted cells are as susceptible to HPV-infection as their surrounding recipient-microenvironment (Figure 5.9 A,B, pg.195).

5.4.4.6 Engrafted cells are not proliferative

The proliferation marker Ki-67 was used to identify actively dividing cells within the sections (Figure 5.9, pg.195). Immunohistochemistry clearly showed proliferating cells in all tested sections. Although Y chromosome-positive cells were observed in the tested sections, they were never found to express Ki-67 and thus appeared not to be proliferative.

5.4.5 The engraftment in different tumour components

An overall comparison between the contributions of donor-derived cells to different tumour components and the normal epithelium revealed that the average percentage of contributing cells is similar within the tumour epithelium and normal epithelial cells, amounting to averages of 4.7±0.8% and 6.5±1.2% respectively. However the contribution of Y-positive cells to stromal myofibroblasts was 14% and thus, significantly different to the tumour ($P<0.0001$) and normal epithelium ($P=0.001$) (Figure 5.10, pg.196).

5.5 DISCUSSION

5.5.1 Donor cell engraftment in different types of post-transplant skin tumours

In this study, we have shown that donor-derived cells stemming from solid organ transplants can engraft into post-transplant skin tumours to varying degrees. Of 176 post-transplant examined skin tumours, 18.75% showed evidence of Y-chromosomal genetic material, distributed among 46% of the tested renal transplant recipients. If Y chromosomal DNA was detected in one lesion, typically, other tumours of the same patient also showed donor cell-incorporation. We showed that presence of donor-derived cells is not dependent on tumour type or degree of malignancy, which is consistent with observations from a previous smaller-scale study by Aractingi *et al.* (2005), who found Y-chromosomal DNA in approximately 50% of examined tumours with no significant difference between different tumour types.

5.5.2 The percentage contribution of Y-positive cells to entire tumour sections

Through highly sensitive qPCR, it was possible to detect as little as 4/15,000 Y-chromosome positive cells. The resulting average frequency of Y-positive cells in the total cell mass of the examined tumour sections ranged between 0.024% - 10.68%, which is in accordance with previous studies investigating donor cell engraftment in the healthy epidermis after BM transplantation in mice (Brittan *et al.* 2005). When the contributing percentages of Y-positive cells were compared among the different tumour types, a significantly higher quantity of Y-positive cells could be detected in malignant compared to non-malignant tumour types. These results stand in contrast to findings from the previously mentioned study by Aractingi *et al.*, which could not detect a difference in engraftment between the different tumour types. The signifantly higher number of tumours studied in our project likely represents a more accurate reflection and statistical relevance of this issue.

Notably, our results were obtained by performing PCR on extracted DNA from entire tumour sections, which includes several different cells types, including stromal, inflammatory, dermal and adipose layers, as well as healthy epidermis. Thus, to more accurately examine the engraftment of Y-positive cells with respect to preferential location and tumour components, histology-based examination methods were used, such as immunohistochemistry and FISH.

5.5.3 Location and cell-type specificity of engrafted Y-positive cells

All tumours that upon qPCR presented with a percentage of Y chromosome-containing cells of >1% were examined by XY-FISH and invariably showed Y-chromosome-containing cells incorporated as single cells, interspersed throughout the sections. In no instance could we find evidence for cell fusion by cells containing more than one Y chromosome. Cell fusion has been implicated as a mechanism for BM cell-engraftment in the liver, but has not been observed in most other epithelia. Even, if fusion events were followed by reduction divisions, this would likely not be

completed in all cells and traces of such a mechanism, such as incomplete resolution should be apparent in some of the examined cells. Thus, we conclude that fusion is not the mechanism by which Y-positive cells engraft into post-transplant skin tumours of female recipients of male kidney allografts.

The dynamics of tissue hierarchy imply that clone sizes of donor-derived cells reflect the hierarchical position of the original engrafted cell. That is, if a cell engrafts and acts as a stem cell, it is capable to give rise to larger and more diverse clones of descendant cells than more committed transit amplifying cells, and logically, if it incorporates as a terminally-differentiated cell, it will remain a single cell of donor-origin in the tissue. Thus, according to our observations, donor-derived cells contribute as terminally differentiated cells to the tumour mass and tumour stroma of post-transplant skin tumour patients.

5.5.4 The donor-origin of engrafted cells

The infrequent and dispersed nature of the Y-positive cells rendered it a difficult task to confirm whether the origin of the engrafted cells lies within the transplanted organ. Previous blood transfusions from male donors could have resulted in haematopoietic chimerism in the patients and be the source of Y-positive cells within the tumours. It has also been argued that foetal microchimerism from previous male pregnancies could result in the systemic distribution of Y-positive cells in the transplant patient and thus result in the engraftment in multiple tissues. In fact, male cells originating from male pregnancies as long as 20 years earlier have been detected in the liver and other tissues of females (Johnson *et al.* 2002, O'Donoghue *et al.* 2004). Previous studies have only marginally dealt with this issue. Bodo *et al.* (2005), proved the donor-derived nature of an entire leukemic clone in a liver-patient by immunohistochemistry for a donor-specific HLA-antigen, but other studies claiming the donor-derived nature of entire post-transplant tumours were unable to prove this, due to unavailability of donor information.

However, several studies used the epidermal engraftment of Y-positive cells in transplanted female/female transplant patients with a known history of previous male pregnancies as a control and could not detect any Y-positive cells (Jiang *et al.* 2004). Additionally, several recent studies have shown that haematopoietic microchimerism is not a frequent event after peripheral blood transfusions and mostly transient and restricted to patients with graft-versus-host-disease (GvHD) (Collins *et al.* 1993).

To test for the occurrence of microchimerism and additionally to exclude infiltrating leukocytes from the cell counts of incorporated cells, we examined CD45-expressing cells for Y-positive cells. The number of Y-positive cells within this population was extremely low, indicating that microchimerism from previous obstetric history or blood transfusions was not present in the tested samples and thus, any Y-positive cells in the tumours are likely from the transplanted organ.

These findings are also supported by our observations on CD34-expressing cells. However, CD34-expression is associated with endothelial cells, as well as with stem cells. In the haematopoietic system, it has been known for many years that CD34 is a stem cell marker (Berenson *et al.* 1988). It also marks dermal stem cells residing in the bulge of the hair follicle in canine and murine skin (Tumbar *et al.* 2004, Pascucci *et al.* 2006). Moreover, recently it was shown that CD34- expression by hair follicles is required for skin tumour development in mice (Trempus *et al.* 2007).

The fact that we could not detect Y-positive cells among the single CD34-expressing cells – possibly representing haematopoietic stem cells – may indicate an absence of haematopoietic chimerism in these patients and also suggests that engraftment does not occur in CD34-positive stem cell niches in the skin.

5.5.3 Cell type-specific engraftment

Previous research has examined the engraftment in epithelial or stromal tumour compartments, but rates of engraftment were never directly compared and differences between cell lineages have never been established. Combined immunohistochemistry and Y-FISH, together with morphological examination helped us to determine that donor-derived cells incorporate into epithelial cells in skin tumours and the normal epidermis, as well as into stromal myofibroblasts. Infrequently, donor-derived cells were seen incorporated into blood vessels. However, no evidence has been found for the incorporated cells to proliferate and thus to contribute to tissue homeostasis on an active and permanent basis.

According to our results, there is a preference for donor-derived cells to engraft into the tumour stroma compared with epithelial cells. This could either reflect a differential affinity resulting in the migration of donor-cells to stromal cells as a response to biochemical cues, or it could represent an effect of different rates of cellular turnover.

We also observed a significant difference in the amount of donor-derived cells between malignant and premalignant tumour epithelial cells, and interestingly, the higher engraftment seen in malignant skin tumours is also observed in the normal skin. The reason for this phenomenon is unclear, but may also lie in different biochemical cues triggering engraftment or cellular turnover.

5.5.4 Mechanisms for donor cell-engraftment in post-transplant skin tumours

Several pieces of evidence exist in the literature reporting on post-transplant tumours that appear to be completely donor-derived. There are several possible mechanisms for the development of these donor-derived cancers. 1) Donor-derived cells transform to become cancer progenitors prior to entering the host; 2) donor cells undergo neoplastic transformation

in the host and then engraft in the skin as cancer-originating cells; 3) donor-derived cells transform after their engraftment into stem cell niches in the skin and adopt cancer stem cell characteristics influenced by their new immediate environment, including HPV infections.

An important study by Houghton *et al.* (2004) provides evidence for the latter scenario. In mice, chronic infection with *Helicobacter felis* resulted in a completely BM-derived gastric cancer, which suggested to the authors that chronic inflammation caused ablation of tissue-resident stem cells, whereupon they are replaced by BM-derived cells, which then, acting as the new tissue-resident stem cells, have the sufficient lifespan to be transformed into CSCs. This indicates that chronic inflammation and the loss of tissue stem cells is a prerequisite for the occurrence of donor-derived tumours. Indeed, acute inflammation and transient injury did not result in donor-derived tumours in this mouse model. Although HPV-infection was a common occurrence among the post-transplant tumours in our study, this viral infection does not result in inflammation and detrimental tissue degradation resulting in stem cell loss.

In humans, although the engraftment of donor-derived cells has been repeatedly reported, very few convincing examples exist of donor-derived tumours. Most donor-derived malignancies were of haematopoietic nature. In the haematopoietic system, stem cells do not home to a locally restricted niche which may facilitate the engraftment of donor cells as stem cells and thus as potential long-lived transformation-prone cancer stem cells. Aractingi *et al.* provided the first and only observation of a Y-positive solid tumour in a kidney transplant patient (Aractingi *et al.* 2005). However, no donor-information or information regarding potential blood microchimerism in the patient was available.

It is difficult to determine the cell type of origin of the engrafted cells. Most studies reporting donor-derived cells in post-transplant tumours report of BM-transplant recipients. The ability of haematopoietic cells to move freely systemically makes them likely candidates for transdifferentiation and

engraftment. Engrafted cells after solid-organ transplants could either stem from cell populations within the grafted organs capable of plasticity, or – more likely- from haematopoietic cells carried over with the grafted organ.

We thus propose a mechanism, whereby haematopoietic cells can be carried over with the grafted organ where they may transiently circulate within the blood stream and thoughout tissues as infiltrating leukocytes, thereby achieving a systemic distribution. Microenvironmental cues can trigger these cells to explore their plastic potential and to transdifferentiate into the cell type with which they are surrounded. In this fashion, donor-derived cells are capable of sporadically engrafting into various tissues of the transplant recipient and contributing to tissue reconstitution by responding to environmental cues and mimicking the cell types that are needed at that particular moment. Cogle and colleagues recently made a similar observation, also reaching the conclusion that the engrafted cells may behave in the form of cell mimickry, rather than playing an active and dynamic part in the tissues they engraft in (Cogle *et al.* 2007).

5.6 CONCLUSION

In summary, our comprehensive study to investigate donor cell-engraftment into skin tumours of kidney transplant recipients revealed that donor-derived cells engraft into tumour and normal epithelial cells and the tumour stroma, whereby the stromal contribution is markedly enhanced. We could not detect peripheral blood microchimerism as evidence for previous blood transfusions or male pregnancies and thus inferred the donor-derived identity of the engrafted Y-positive cells. Donor-derived cells were only seen as sporadically-distributed cells, never seen in clusters or as proliferating cells, and thus we conclude that in a normal setting, in the absence of chronic inflammation, donor-derived cells from kidney transplants are not likely to engraft as stem cells when triggered to engraft into the skin or skin tumours of the recipients.

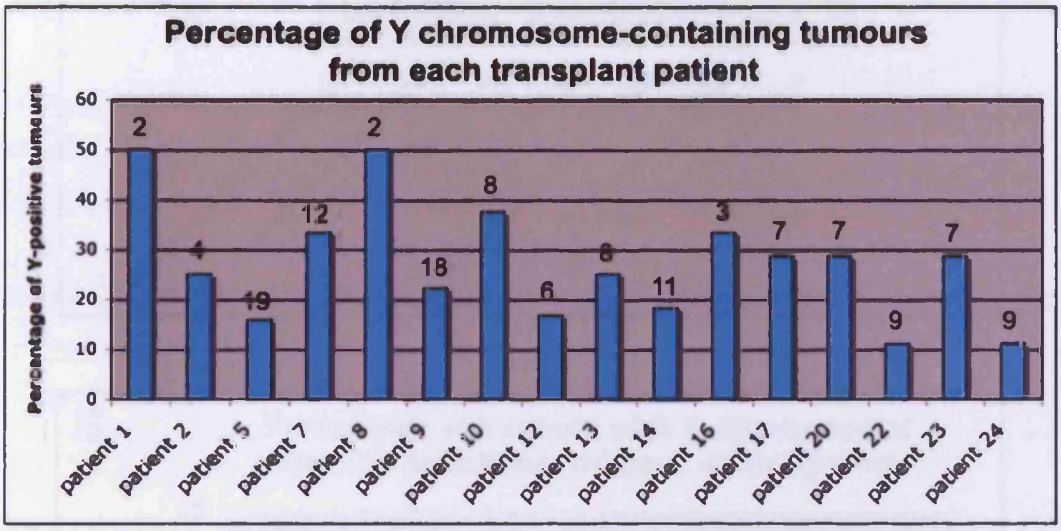
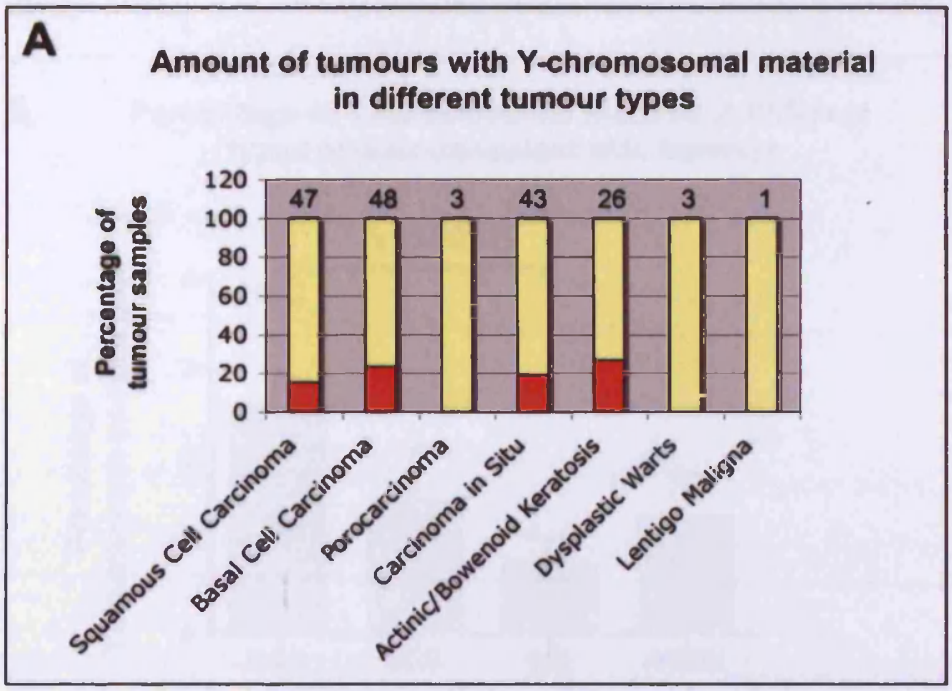


Figure 5.1a. The percentage of post-transplant skin tumour samples in which Y-chromosomal material was detected for each patient who displayed Y-containing tumours. Y chromosomal DNA was determined by qPCR for *SRY*-specific sequences. Number of samples is indicated above bars.



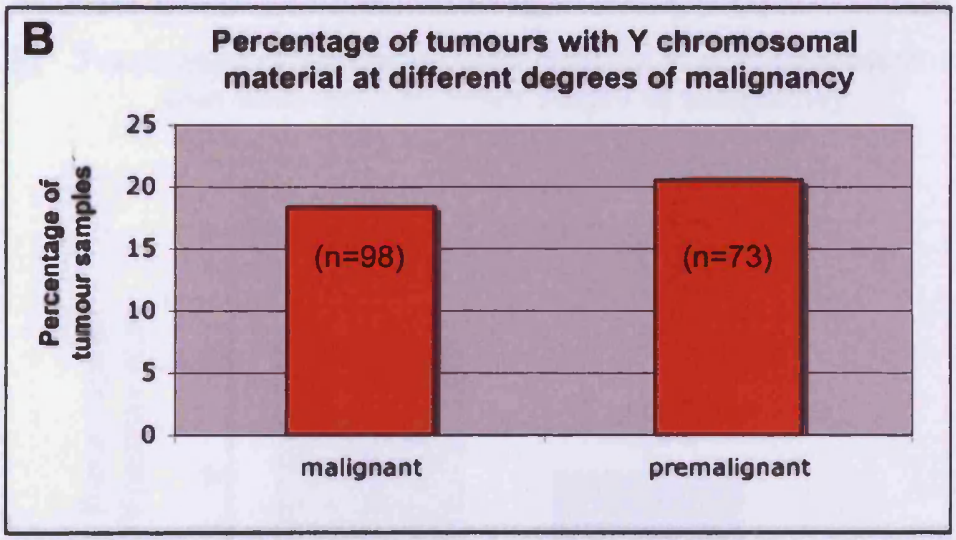


Figure 5.1b. (A) Percentage of tumours containing Y-chromosomal material in different post-transplant tumour types. Red segments indicate Y-positive percentage, yellow segments indicate Y-negative percentage. (B) Percentage of tumours containing Y-poitive cells according to stage of of tumour development. Number of samples are indicated above bars.

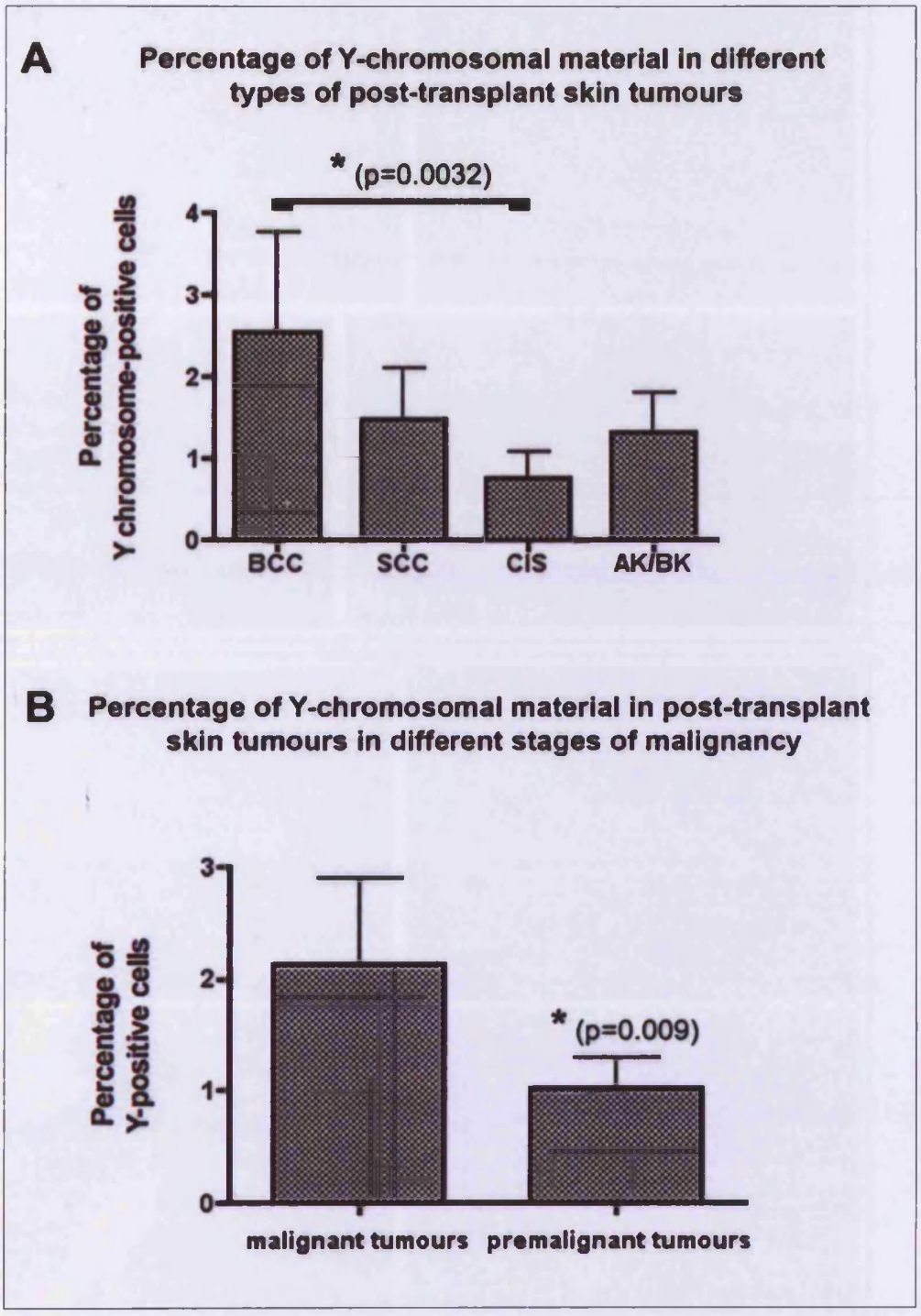


Figure 5.2. (A) Percentage of cells containing Y chromosomal DNA in different types of post-transplant skin tumours. (B) Percentage of cells with Y-chromosomal DNA in malignant and premalignant tumours. Amount of Y chromosome-positive cells determined per 50 ng of DNA. Asterisks indicate statistical significance.

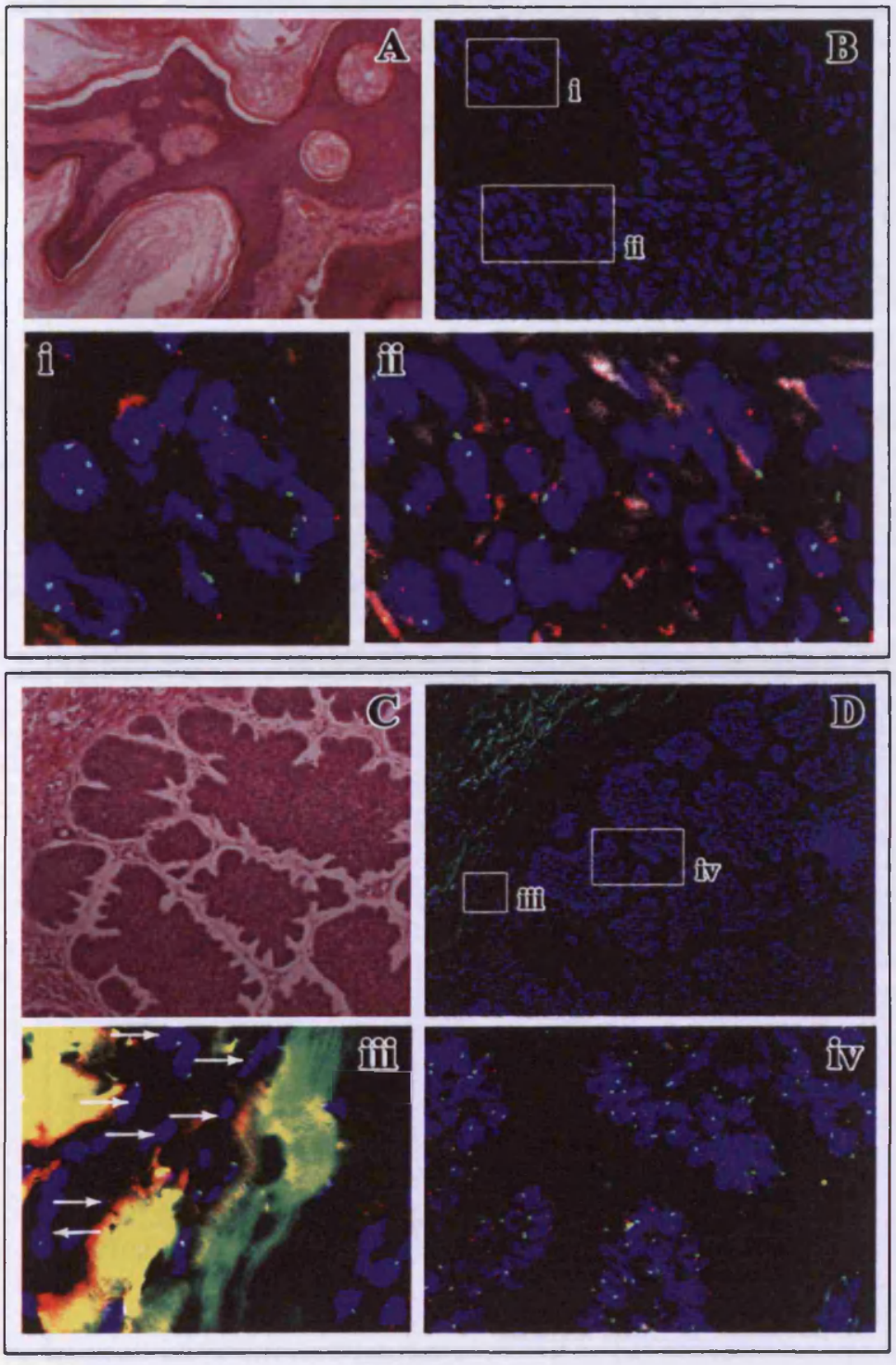


Figure 5.3. (A,B) SCC of a male patient. (C,D) BCC of a male patient. (A,C) H&E staining confirming histology. (B,D) Sections showing DAPI-stained nuclei. (i,ii,iii,iv) Tumour areas marked by white squares in (B), morphologically representing (i,iii) tumour stroma and (ii,iv) epithelial tumour components shown in higher magnification to visualise sex chromosomes detected by XY-FISH. Y-chromosomes visualised by red probe, X-chromosomes visualised by green probe.

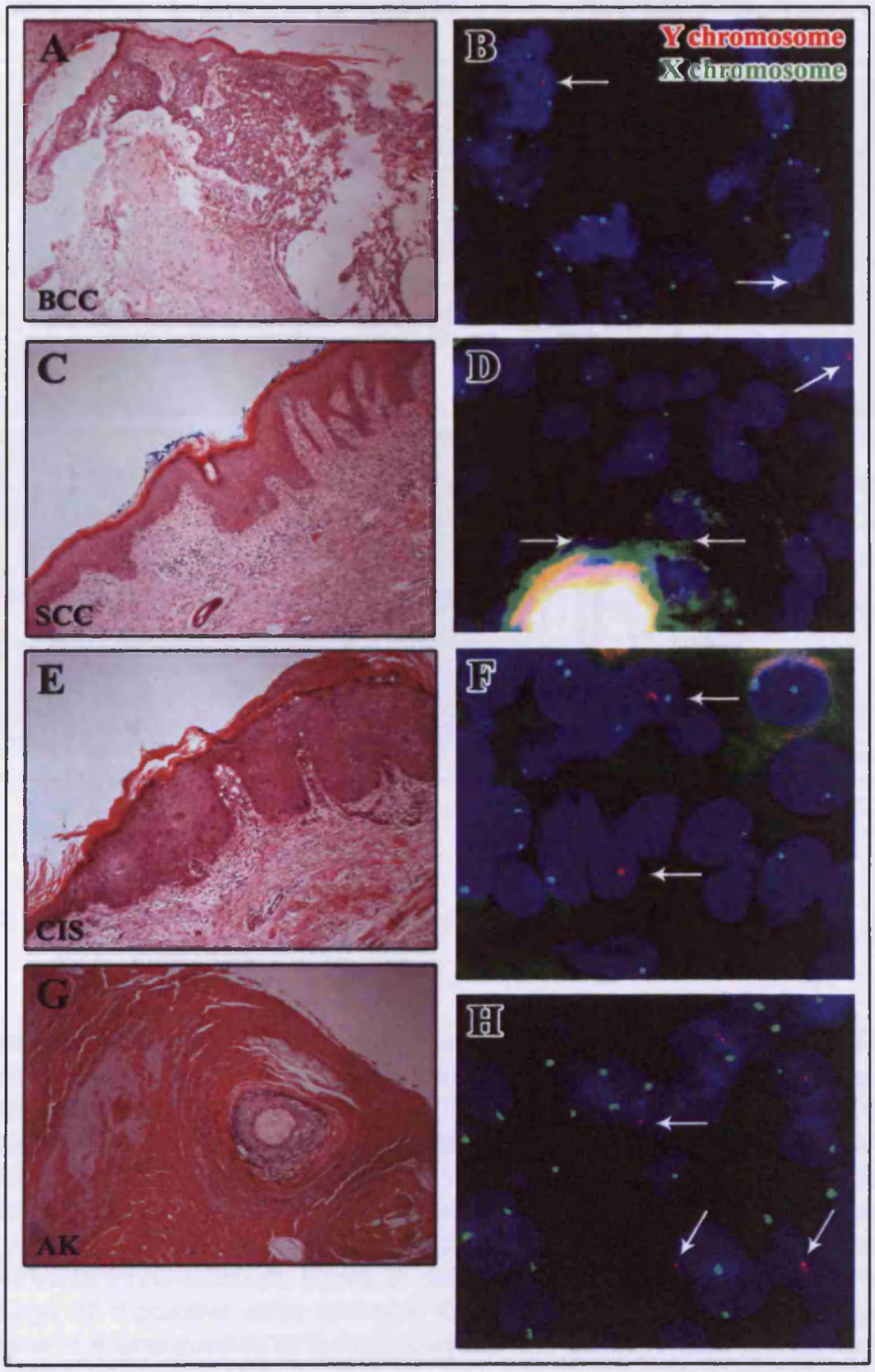


Figure 5.4. (A,C,E,G) H&E stained sections of different tumour types containing Y-positive cells. (B,D,F,H) XY-FISH on corresponding serial sections to visualise cells containing Y chromosomes. Y-chromosome visualised by red probe, X chromosome visualised by green probe. No cell fusion is observed.

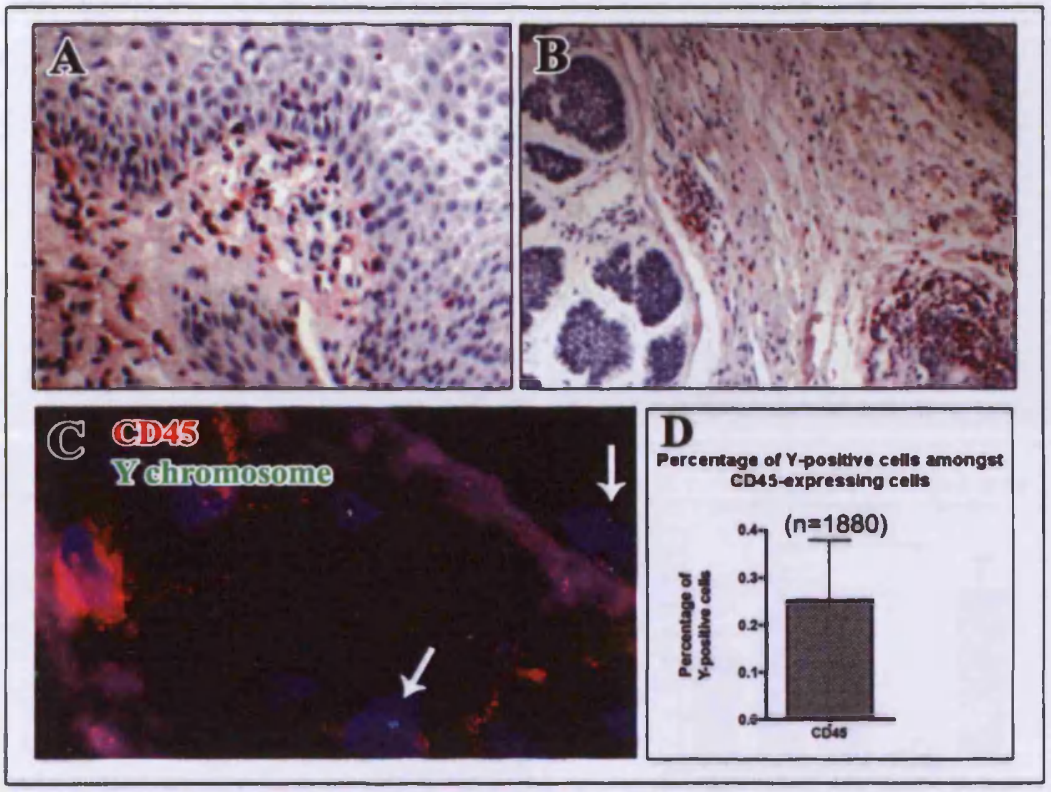


Figure 5.5. (A, B) Immunohistochemistry for CD45 to show location of infiltrating leukocytes. CD45-positive cells, labelled by vector red, appear in distinct compartments (A) within and (B) outside the lesions and are of different morphology than the large epithelial cells comprising the lesions. (C) Combined immunhistochemistry and Y-FISH shows Y-chromosome positivity among some of the CD45-positive cells (arrows), in an area distinct from the Y-positive CD45-negative cells of different morphology. Y chromosome probes are FITC-labelled, CD45 is detected by vector red (red). (D) Percentage of Y-positive cells amongst CD45-expressing cells. A correction factor of 1.4 was applied to the counted cell numbers to reach final percentage.

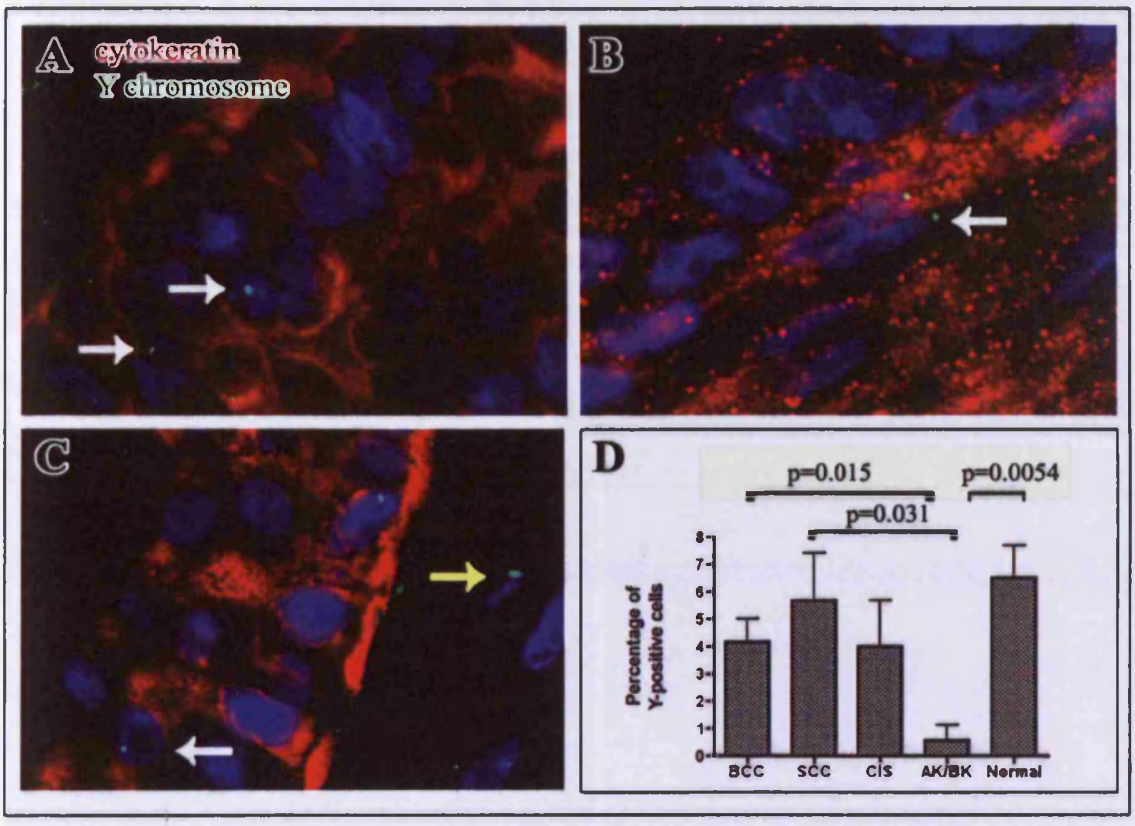


Figure 5.6. Combined fluorescent immunohistochemistry for cytokeratin (red) and Y-FISH (green) to show donor cell engraftment in epithelial cells in (A) squamous cell carcinoma (SCC); (B) normal epithelium; (C) basal cell carcinoma (BCC). Double-positive cells are marked with white arrows. Note the donor-derived cell of elongated morphology in the adjacent cytokeratin-negative tissue component in (C) (yellow arrow). (D) Percentage of engrafted donor cells among cytokeratin-positive cells in different types of post-transplant skin tumours and normal epithelium. Differences in engraftment between BCC, SCC, and carcinoma *in situ* (CIS) are not significant, but are statistically significant compared to actinic and bowenoid Keratoses (AK/BK). P values are indicated where statistical significant differences apply

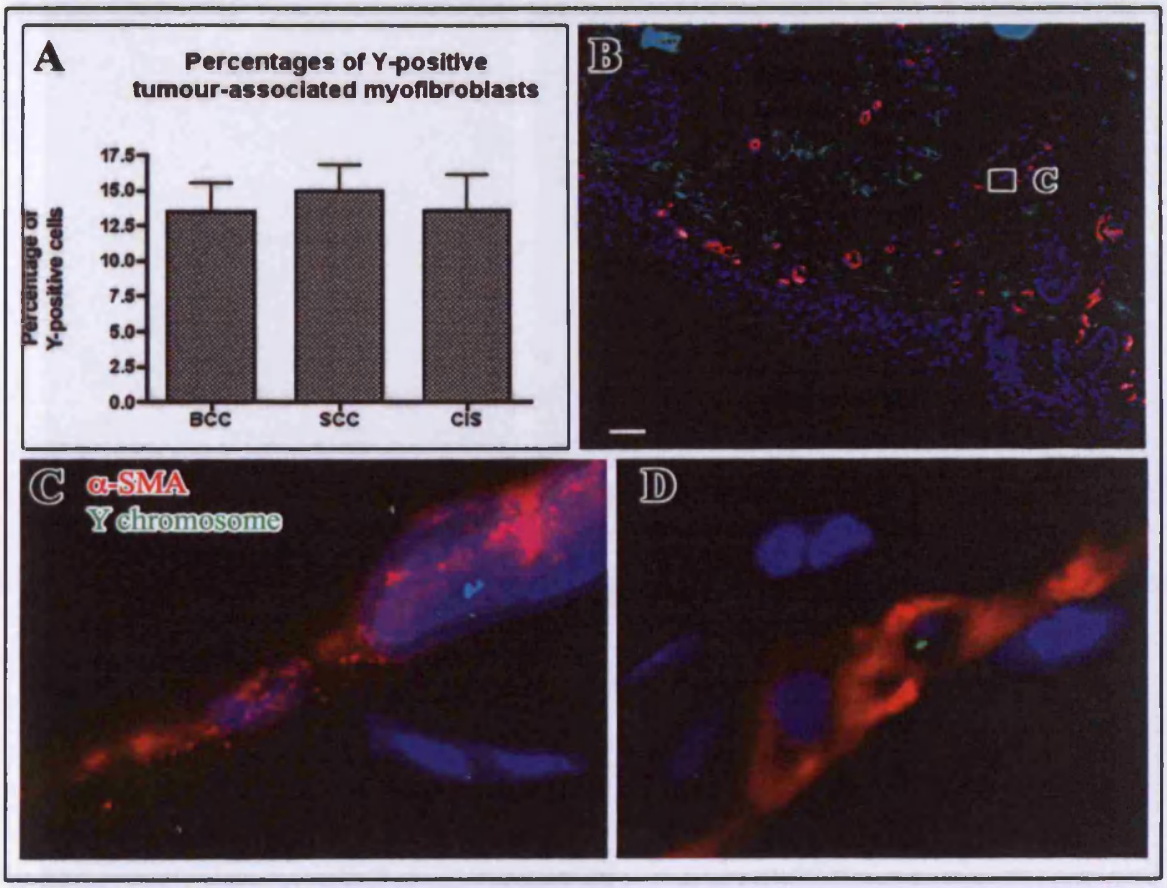


Figure 5.7. Combined immunohistochemistry for α-SMA (red) and Y-FISH (green) to show donor-derived stromal myofibroblasts. (A) Percentages of Y-positive myofibroblasts amongst different types of post-transplant skin tumours. Differences are not statistically significant. (B) Fluorescent immunohistochemistry showing α-SMA-positive myofibroblasts and DAPI-stained nuclei. (C) Magnification of area indicated by white square in (B) showing Y-positive myofibroblast. (D) Double-positive cell from a different post-transplant skin tumou

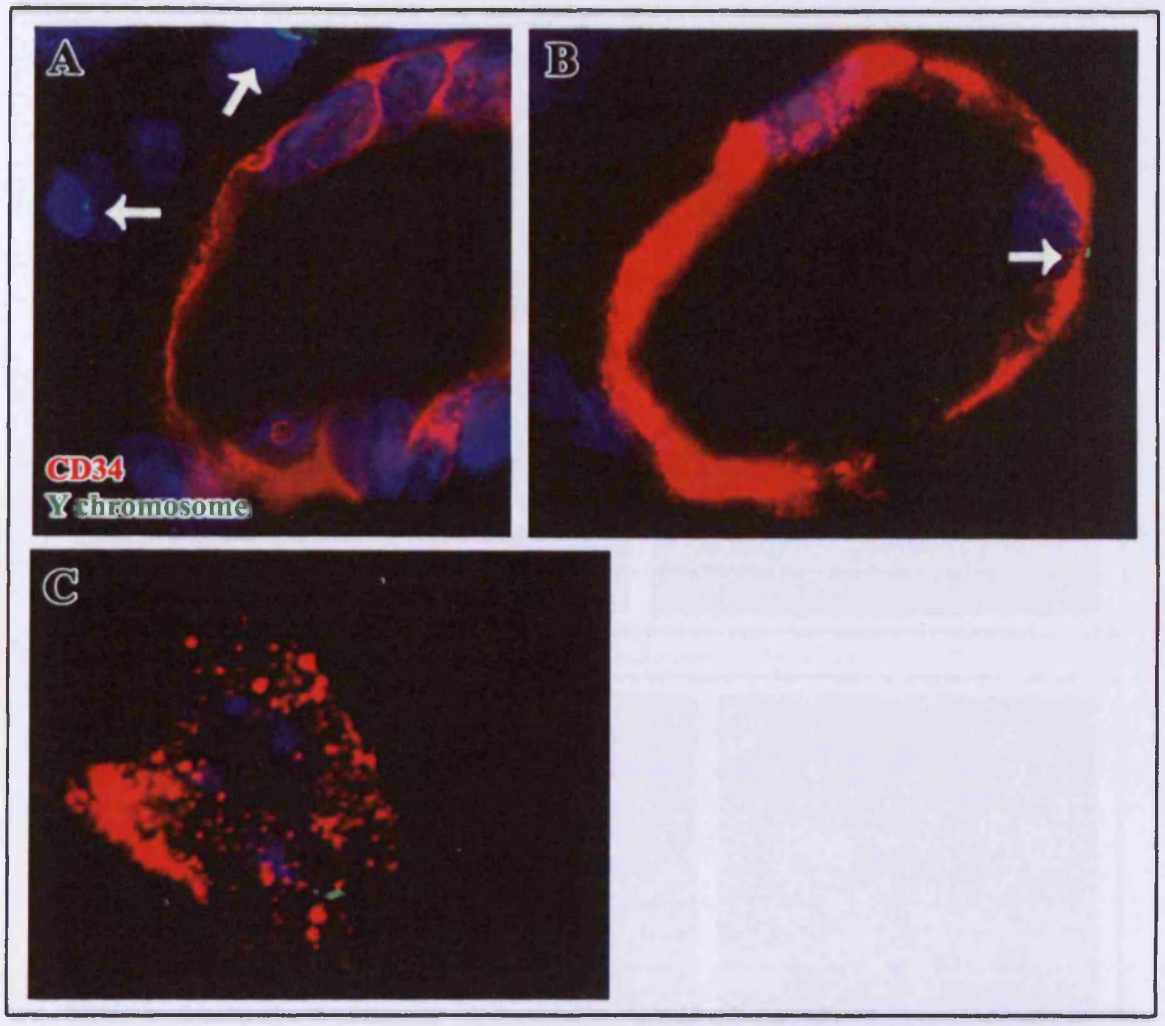


Figure 5.8. Combined immunohistochemistry for CD34 (red) and Y-FISH (green). (A) Typical staining profile. Y-positive cells are seen in the surrounding structures (indicated by white arrows), but very rarely amongst CD34-expressing cells. (B) Double-positive cell within vessel-like structure (indicated by white arrow). (C) Double-positive sporadic cell in male control, not appearing to be part of a blood vessel.

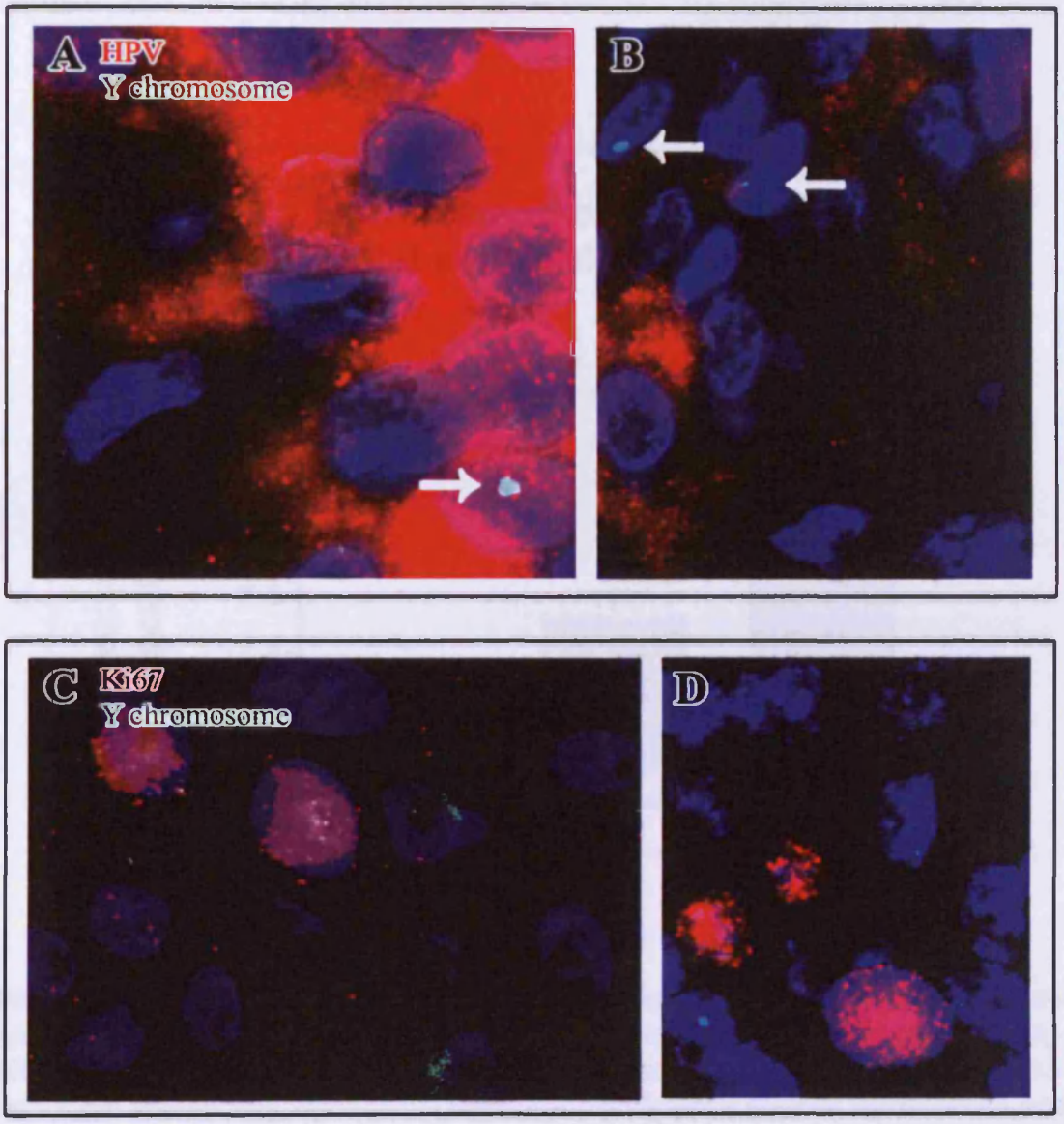


Figure 5.9. (A,B) Two representative sections of combined immunohistochemistry for HPV (red) and Y-FISH (green) to show donor-derived HPV infected cells. Y-positive cells are observed sporadically in HPV-infected areas. double-positive cells are marked by white arrows. (C,D) Combined immunohistochemistry for Ki-67 (red) and Y-FISH (green) to show proliferating donor-derived cells. No double positive cells were found. Note the surrounding Ki-67-negative cells.

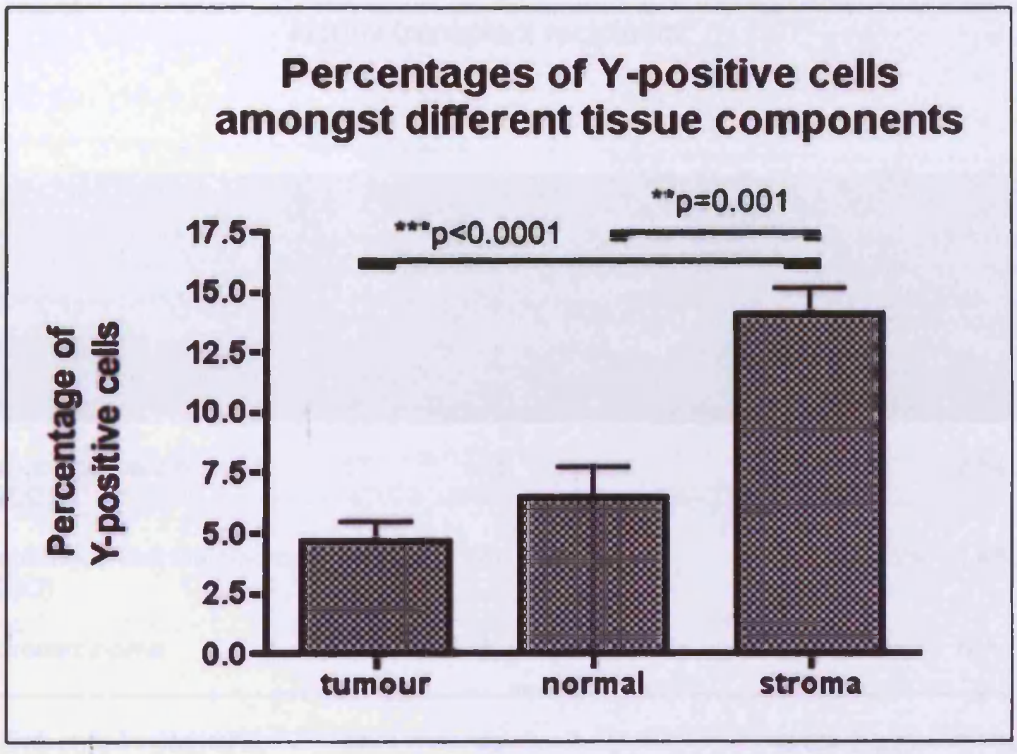


Figure 5.10. Percentage of Y positive cells among various tissue compartments. differences between tumour and normal epithelium is not statistically significant, wheras the differences between the stroma and the other tissue components are statistically significant (statistical significance marked by asterisks and p values).

Table 5.1

Distribution of Y-positive cells among different types of skin tumours in kidney transplant recipients

	Type of lesion	Number of patients affected	Total number of lesions	Number of lesions with Y-positive cells	Percentage content range of Y-positive cells	Percentage content of Y-positive cells (mean±SEM)
Malignant	Basal Cell Carcinoma (BCC)	17	48	11	0.05-10.68%	2.54±1.21%
	Squamous Cell Carcinoma (SCC)	23	47	7	0.02- 3.93%	1.48±0.62%
	Porocarcinoma	3	3	0	N/A	N/A
Pre-malignant	Carcinoma *in situ* (CIS)	17	43	8	0.06- 2.32%	0.76±0.31%
	Actinic/Bowenoid Keratosis (AK/BK)	16	26	7	0.09- 3.52%	1.32±0.48%
	Lentigo Maligna	1	1	0	N/A	N/A
	Dysplastic Warts	3	3	0	N/A	N/A

CHAPTER VI

THE EFFECT OF LOCAL MICROENVIRONMENT AND GENETIC PREDISPOSITION ON CANCER DEVELOPMENT IN A MOUSE MODEL OF SKIN CARCINOGENESIS

6.1 INTRODUCTION

6.1.1 BM transplantation and plasticity

The plasticity of transplanted BM cells and their capability to engraft into multiple tissues has been well documented in mice, as well as in humans and is described in more detail in section 1.8. The factors, which trigger BM cells to transdifferentiate and home to the new microenvironment are unknown. Nevertheless, the fact that stem cell plasticity is also observed in adult tissues challenges the notion that stem cell pluripotentiality is a feature exclusive to embryonic stem cells. This presents a new outlook on regenerative medicine as adult stem cells are devoid of the ethical difficulties associated with the use of their embryonic counterparts.

The extent of engraftment varies between different studies and tissues, is enhanced by injury and appears to reflect the turnover-rate of the tissue of destination. Cell fusion has been attributed to the mechanism involving BM cell incorporation in some cell lineages, such as hepatocytes, skeletal and cardiac muscle, epithelial and Purkinjie cells (Wang *et al.* 2003, Willenbring *et al.* 2004, Camargo *et al.* 2003, Nygren *et al.* 2004, Spees *et al.* 2003, Weimann *et al.* 2003), but these observations have been subject to debate and could not be confirmed in most other tissues.

The BM contribution to the normal mouse skin is 1-7%, appearing as sporadic cells without any evidence for cell fusion (Krause *et al.* 2001, Brittan *et al.* 2005, Korbling *et al.* 2002, Badiavas *et al.* 2003, Harris *et al.*

2004, Kataoka *et al.* 2003). In areas of injury, engraftment is enhanced up to 13% and has been observed in known stem cell niches such as the epidermal proliferative units (EPU) of the skin and in distribution patterns that suggest an origin in a common ancestor, i.e. a stem cell (Fathke *et al.* 2004, Brittan *et al.* 2005).

If transplanted cells engraft as stem cells in a tissue, they potentially have the longevity and proliferation capacity to form fully donor-derived tumours. In accordance with this theory, Houghton *et al.* (2004) showed BM-derived gastric cancer in a mouse model of chronic inflammation. Tumours entirely composed of donor-derived cells have also been reported in humans. Donor-derived haematological malignancies have been seen after BM and solid organ transplantation (Papadopoulos *et al.* 1994, Bodo *et al.* 2005, Barozzi *et al.* 2003). One case of a donor-derived solid tumour (BCC) after solid organ (kidney) transplantation has been described (Aractingi *et al.* 2005). However, unavailability of donor-specific information made it impossible to rule out an origin in haematopoietic cells stemming from earlier blood transfusions. In contrast with these observations, other researchers never found fully donor-derived tumours post-transplant neoplastic lesions in either humans or mice, and suggested that the sporadic contribution of donor-derived cells represents a form of cell mimicry by the donor-derived cells (Cogle *et al.* 2007).

6.1.2 Effect of local microenvironment on transplanted cells

The incorporation of transplanted cells into various tissues of the recipient raises the question to what extent the incorporated cells may be influenced by their new immediate environment and *vice versa*. For example, if the incorporated stem cells carry a genetic predisposition to neoplastic transformation, are they able to engraft into the tissue of destination as cancer stem cells and generate a cancer in the host? Similarly, in a pre-disposed host, will the engrafted cells merely transform as part of the cancerous lesion or are they able to influence the local surroundings as a result of the normal genetic information they carry? If genetic predisposition

in a transplanted cell has an impact on the host, a new risk associated with transplantations is presented. On the other hand, an immediate influence of incorporated cells might also facilitate new exciting therapeutic approaches.

The immediate cellular environment with its biochemical cues clearly exerts a strong influence on an engrafted BM-derived cell. It primes the cell to adopt new functions inherent to the new resident tissue and in the case of stem cells to give rise to differentiating cells. The impact of the immediate cellular environment is highlighted in the event of chronic inflammation, where it has been shown that intercellular factors such as adhesion molecules or cytokines are ultimately responsible for malignant transformation.

Importantly, transplanted cells have also affect their immediate surroundings. In a mouse model of colitis, transplanted BM-derived cells were able to alleviate the symptoms and contribute to epithelial healing (Bamba *et al.* 2006). Similarly, in a rat model of myocardial infarction incorporation of BM-derived cells significantly improved cardiac function (de Macedo Braga *et al.* 2007). This suggests that the effects between incorporated cells and their local microenvironment are reciprocal, and similarly challenges the notion of incorporated cells merely contributing to their destination tissue in the form of cell mimicry.

6.2 AIMS

In this study, we aimed to investigate whether genetically predisposed transplanted cancer stem cells are influenced by their immediate surroundings, and to simultaneously investigate whether transplanted normal cells can counteract a genetic predisposition to cancer. Additionally, our intention was to monitor the extent of engraftment of BM cells into the normal and wounded skin, as well as into cancerous lesions. Finally, this study was carried out to test whether there is preferential cell type-specific engraftment and whether the engrafted cells are functional or just mimic their immediate environment.

6.3 METHODS

All methods are outlined in detail in chapter II.

6.3.1 Mouse strains

The mouse strains used for BM transplantations were either C57/ Black 6 wild type (wt) mice or transgenic mice with genomic incorporation of the complete early region of the cutaneous human papillomavirus 8 (HPV8) as described in chapter II, section 2.3.3.1. HPV8 transgenic mice develop single or multifocal epidermal lesions with a median age of tumour development of 8.4 weeks. 91% of the developing tumours are benign with the potential to proceed to moderate or severe epidermal dysplasia. Squamous cell carcinomas (SCC) arise in 6% of HPV8 mice (Schaper *et al.* 2005).

6.3.2 BM transplantation

Six week-old female recipient mice were lethally iradiated, followed by tail vein injection of variable amounts of whole BM cells from male mice. Details of transplanted experimental groups are described in chapter II, Table 2.3. All mice were culled by CO_2 inhalation and cervical dislocation 20 weeks post-transplant or when tumour burden was unbearable (exceeding the threshold diameter of 20 mm or multiple tumour foci). Epidermal lesions, as well as sections of unwounded and wounded skin were fixed in NBF and embedded in paraffin.

6.3.3 Cutaneous wounds

Four weeks post-transplantation, two cutaneous wounds of 3 mm diameter, were made to the dorsal skin of the mice in the groups indicated in chapter II, Table 2.4. Mice were killed 20 weeks post-transplant or when tumour burden was unbearable (exceeding the threshold diameter of 20 mm or multiple tumour foci).

6.3.4 Y-FISH

Sections were dewaxed, permeabilised in 1 M sodium thiocyanate, and digested in 0.4% pepsin at 37°C for 20 min. They were then fixed in PFA and dehydrated through alcohol gradients. A fluorescent probe against the mouse Y-chromosome, directly conjugated to a FITC-label was applied, sections were hybridised at 60°C, incubated overnight at 37°C and mounted in medium containing DAPI.

6.3.5 Combined immunohistochemistry and FISH

Sections were dewaxed and subjected to a three-step immunohistochemistry protocol using the strept-AP detection system. Final detection was carried out with the Vector Red Alkaline Substrate Kit 1 (Vector Laboratories). Primary antibodies used were to detect pan-cytokeratin, SMA, CD31, and Ki-67. The immunohistochemistry protocol was immediately followed by *in situ* hybridisation for the Y-chromosome as described above. Where antigen retrieval was performed during the immunohistochemistry protocol, digestion times were adjusted to 2 min.

6.3.6 Statistical analysis

Statistical analyses were conducted using Mann-Whitney and Kruskal-Wallis analyses with Dunn's post-test for non-parametric data and the Student's two-tailed t-test for parametric data where appropriate (Prism, GraphPad, San Diego). A probability (P) value of ≤ 0.05 was taken as an appropriate level of significance.

6.4 RESULTS

6.4.1 Tumour development

We observed development of skin lesions in HPV8 transgenic mice with an average latency of 9 weeks. This was in accordance with previous reports concerning the HPV8 phenotype. Most lesions developed as multifocal papillomas with the tumour burden exceeding the permitted threshold before they could progress to further degrees of malignancy (Figure 6.1, pg.208).

Tumour development was specific to HPV8 transgenic mice. Wt type mice did in no instance develop skin lesions within the experimental period of 5 months, irrespective of the genotype of BM they received and the post-transplant treatment (Table 6.1, pg.216). The type of BM administered influenced the latency of tumour development significantly (P=0.0411) (Figure 6.2A, pg.209). Unexpectedly, recipients of wt BM showed the first signs of skin lesions on average 3 weeks earlier than recipients of HPV8 BM. This indicates, that the type of donor BM may influence the genetically pre-determined phenotype.

Post-transplant treatment in the form of epidermal wounding also had an impact on the latency of tumour appearance in the tumour-developing HPV8-transgenic recipient mice (Figure 6.2B, pg.209). Groups that were wounded 3 weeks after BM transplantation developed tumours at around 7.8±0.8 weeks, which was significantly earlier than the 10.7±0.9 weeks in non-wounded groups (P=0.041). In wounded mice, tumours preferentially developed in the previously injured area.

6.4.2 Extent and location of engraftment of donor-derived cells

Y-FISH revealed the incorporation of donor-derived cells into the normal skin and skin tumours of BM recipient mice. Engraftment was seen in the normal epithelium, and both the bulk of the skin tumours and the tumour

stroma, mostly in small clusters of cells and as sporadically incorporated cells with a different rate of incorporation in each part of the tissue (Figure 6.3, pg.210). Male control samples showed that approximately 25% of all nuclei could not be labelled by Y-FISH as a consequence of inaccessibility of nuclei due to orientation or cutting. Therefore, a correction factor of 1.3 was applied to account for the potential underestimation upon counting of Y-positive nuclei.

The genetic background of the recipient mice and BM type did not influence the degree of engraftment. Mice that were subjected to epithelial injury showed a significantly higher incorporation of donor-derived cells in all tested tissue components and across all experimental groups (Figure 6.4, pg.211). Donor-derived cells engrafted into normal epithelia distributed sporadically throughout the epithelial layers, contributing 2.5-11% of all epithelial cells. The epithelial components of the skin tumours showed a greater fraction of donor-derived cells, amounting to 6-26%. The highest percentage of donor-cell incorporation was observed in the tumour stroma, with up to 38% of stromal cells showing Y-positivity and thus being of donor-origin. Whereas there were no significant differences between the experimental groups comprising the wounded and non-wounded fractions, wounding had a significant impact on engraftment in all tissue components. After wounding, significantly higher amounts of Y-positive cells contributed to normal epithelia ($P=0.0002$), epithelial tumour cells ($P=0.0022$), and tumour stroma ($P=0.0006$) when compared to the non-wounded counterparts.

6.4.3 Identity of engrafted cells

A technique combining Y-FISH with fluorescent immunohistochemistry against cell-type specific antigens was employed to reveal the identity of the engrafted donor-derived cells, whereby the resulting observations confirmed the results obtained upon Y-FISH and morphological analysis. Y-positive cells of epithelial nature (expressing cytokeratin) were observed sporadically distributed (Figure 6.5, pg.212). Donor-derived cells were also

capable of engrafting as stromal cells, identified through their expression of α-SMA (Figure 6.6, pg.213). Moreover, we observed sporadic donor-cell incorporation in the vascular components of the tumour lesions, marked by their expression of the endothelial surface antigen CD31 (Figure 6.7, pg.214). Ki-67, a protein expressed specifically in actively proliferating cells has also been found to be expressed in donor-derived cells, indicating that the donor-cells engraft as functional and dividing cells (Figure 6.8, pg.215). Notably, all these observations were not dependent on the type of BM administered (i.e. HPV8-positive or wt).

6.5 DISCUSSION

6.5.1 Engraftment of BM cells in epidermis

In this study, we confirmed that transplanted BM cells engraft into the normal epidermis, skin tumours and their surrounding stroma in a functional and proliferating manner in the absence of cell fusion. Expression of cell type-specific surface markers confirmed that the cells fully transdifferentiated, suggesting that the engraftment did not merely represent a form of cell mimicry.

The stromal components showed a higher efficiency of donor cell-engraftment than both the tumour and normal epidermal tissue components. This is consistent with previously published reports, showing preferential BM engraftment into the stroma of pancreatic tumours, but rarely in the tumours themselves (Direkze *et al.* 2004). Although we detected a slightly larger percentage of engrafted cells in the skin tumours than in the normal epidermis, this was statistically not significant.

It is well known that injury increases the rate of engraftment of BM-derived cells. This was confirmed by our results. Epithelial injury also decreased the latency of tumour appearance. However, there was no correlation between wounding and transplanted BM type.

6.5.2 Effect of transplanted cells on tumour development

In the literature, it has been proposed that donor-derived cells stemming from BM transplantations, and even from solid organ allografts, could give rise to entire post-transplant cancers in humans (Barozzi *et al.* 2003, Bodo *et al.* 2005, Aractingi *et al.* 2005). In mice, Houghton and colleagues (2004) have demonstrated BM-derived gastric cancer upon *H.felis* infection. Based on these studies, two possibilities for tumour development were proposed. The transplanted cells could have carried a predisposition for cancer development and thus presented an inherent risk of transplantation, or the transplanted cells could have engrafted in the tissue as sporadic cells, undergoing transformation at the site of engraftment. This raises two important issues: 1) does transplantation of genetically pre-disposed cells present an increased risk for the recipient to develop cancer? 2) Can engrafted healthy cells have a positive effect on their genetically-predisposed surroundings and thus be explored for therapeutic purposes?

In this study, we could not detect an effect of engrafted donor-derived cells on their surroundings. The expected outcome phenotype of the recipient mice was not reversed or decreased by BM transplantation of cells with a different genetic predisposition. For example, although donor-derived HPV8-positive cells engrafted as functional cytokeratin-expressing and actively proliferating cells into the recipients' epithelia, wt mice did in no instance develop skin lesions. It is conceivable that the experimental course of 5 months was not sufficient time to allow the recipient cells to show the full extent of their influence on their surroundings. But interestingly, injection of HPV8 mice with wt BM did not even retard the development of the resultant phenotype. On the contrary, the latency of tumour formation was decreased compared to the control group recipients of HPV8-BM. This might be due to adverse physiological reactions and recognition of the genetically different BM.

We could not detect an adverse effect of engrafted transgenic cells on their new surroundings. Similarly, we could not find reason to suggest that a genetically predisposed environment stimulates transformation of newly engrafted cells to cancer stem cells, giving rise to cancerous clones. In fact, no patterns of donor-derived cells were observed that would suggest common ancestry, which renders it unlikely that donor-derived cells engrafted in stem cell niches or assuming stem cell function. The absence of entire tumours or larger clones derived from donor-derived cells indicated that genetic predisposition might not present an additional risk factor to transplantation medicine. Additionally, this suggests that external factors such as UV exposure are responsible for triggering transformation after engraftment.

6.6 CONCLUSION

It has been previously suggested that donor-derived cells could be the source of tumours in mice and in humans. We could not observe donor-derived skin tumours in our present transgenic mouse model of spontaneous skin carcinogenesis with or without the effect of acute skin injury. Our results suggest that transplanted cells show considerable plasticity and can engraft into tissues distant to their site of origin as functional members of the target tissue. There they may be capable of undergoing neoplastic transformation and act as cancer stem cells, particularly if they engrafted into the tissue as stem cells. However, we could not find evidence for the hypothesis that the genetic make-up of transplanted cells exert an effect on their surroundings and thus present an additional risk factor for transplant patients. Further research involving long-term studies is necessary to confirm these results.

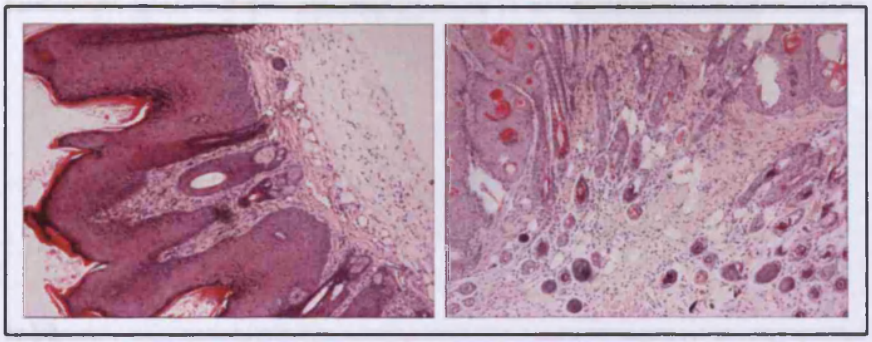


Figure 6.1. H&E stainings showing epithelial tumours typically developed by HPV8-transgenic mice.

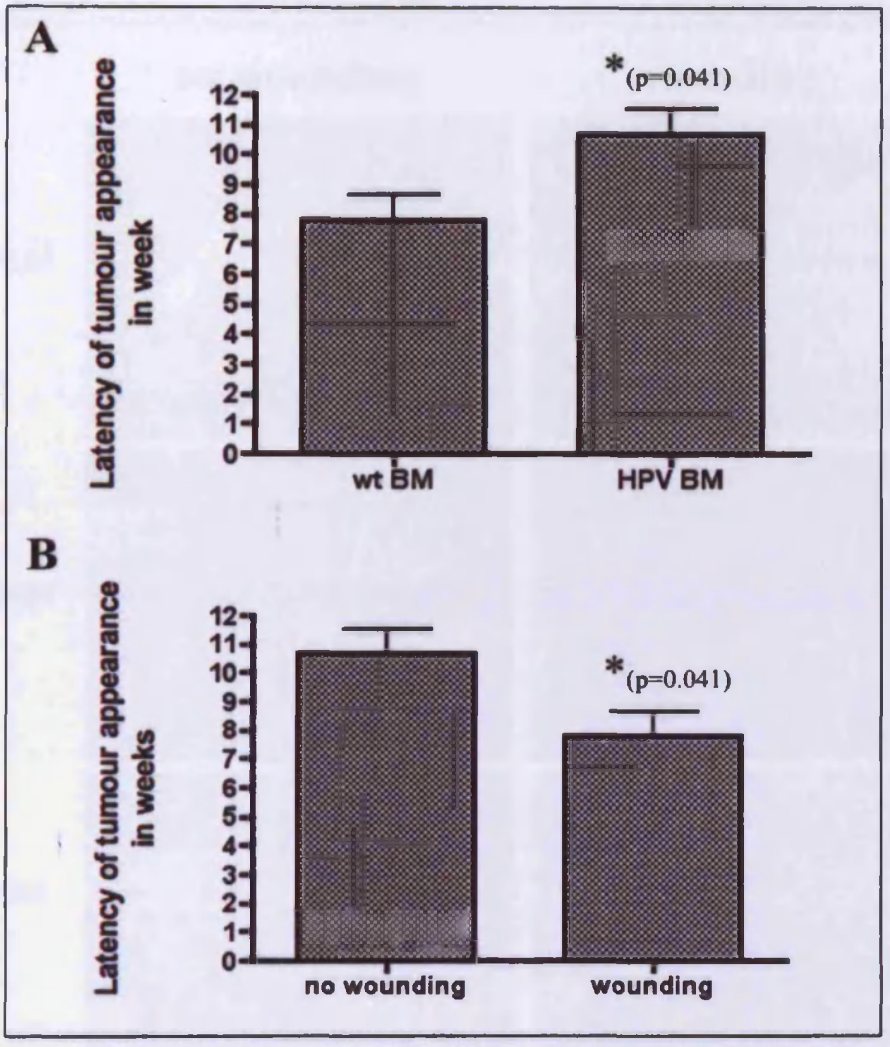


Figure 6.2. Latency of tumour development in HPV8 transgenic mice measured in weeks after BM transplantation. (n=6 for each group). (A) Differences in tumour latency were significant between HPV8 transgenic mice depending on administered BM (P=0.0411). Compared to the control groups (HPV mice receiving HPV BM), receiving wt BM decreased tumour latency. (B) Wounding significantly decerased the tumour latency in HPV8-transgenic mice (P=0.0411). Note that no tumour development occured in wt recipient mice, hence these groups are absent from this graph. X axis legend indicates 'BM recipient/BM donor .

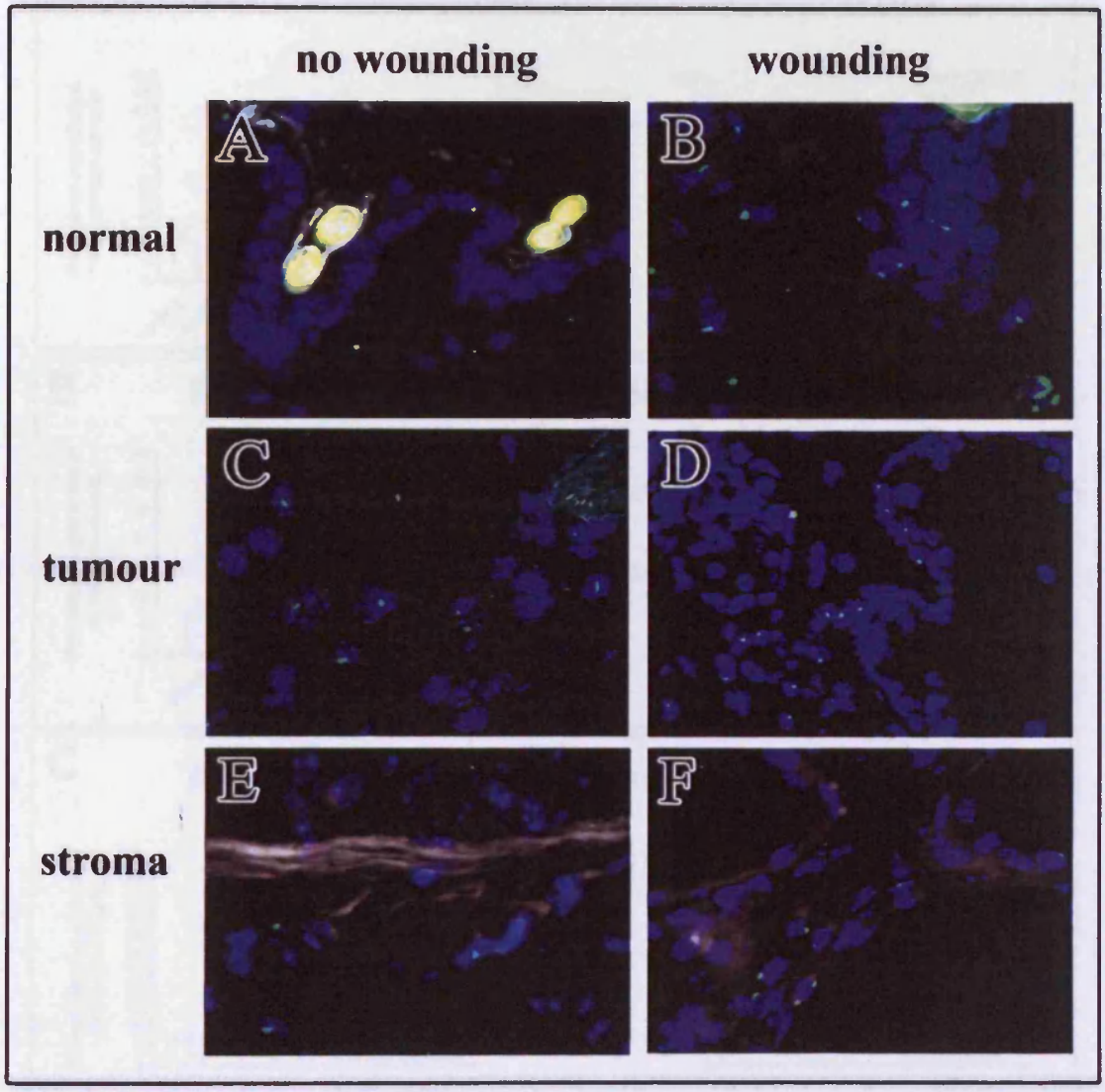


Figure 6.3. Engraftment of donor-derived cells into various tissue components. The Y chromosome is detected by green fluorescent FITC-labelled probe. Donor-derived cells were found in (A,B) normal epithelia, (C,D) tumour bulk, (E,F) stromal components. Nuclei were counterstained with DAPI (blue).

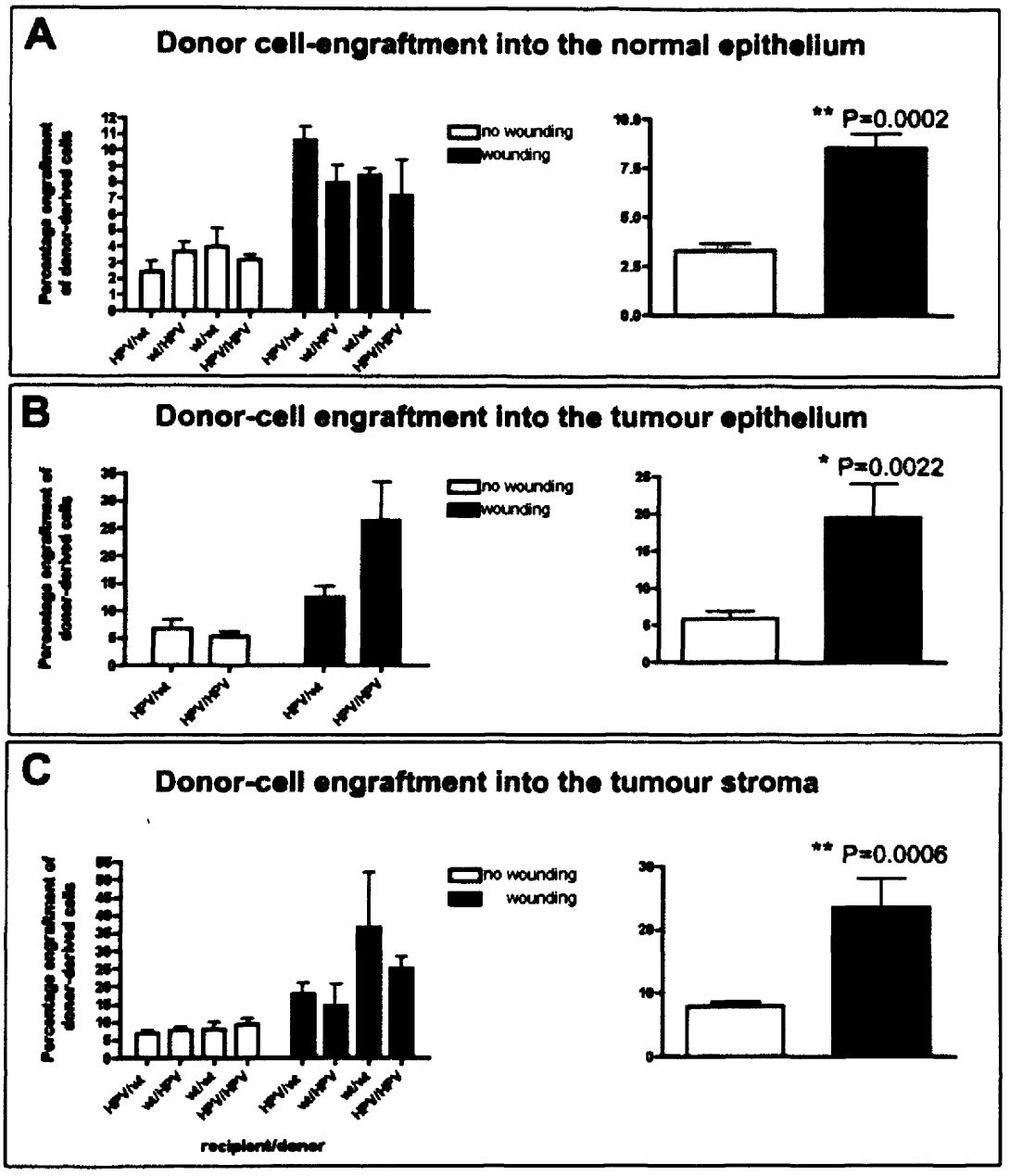


Figure 6.4. Engraftment of donor-derived cells into various tissue components with and without epithelial injury. Across all groups, wounding increased the percentage of engraftment significantly (significant differences marked by asterisks and P values). Within the wounded and non-wounded groups, no significant differences were found. Collective assessment of wounded and non-wounded groups are shown on right hand graphs, displaying significant differences. (A) Percentage contribution of donor-derived cells to the normal epithelium. (B) Percentage contribution of donor-derived cells to epithelial tumour cells. (C) Percentage contribution of donor-derived cells to the tumour stroma. Overall, more engraftment was seen in the tumour stroma than in the other tissue components.

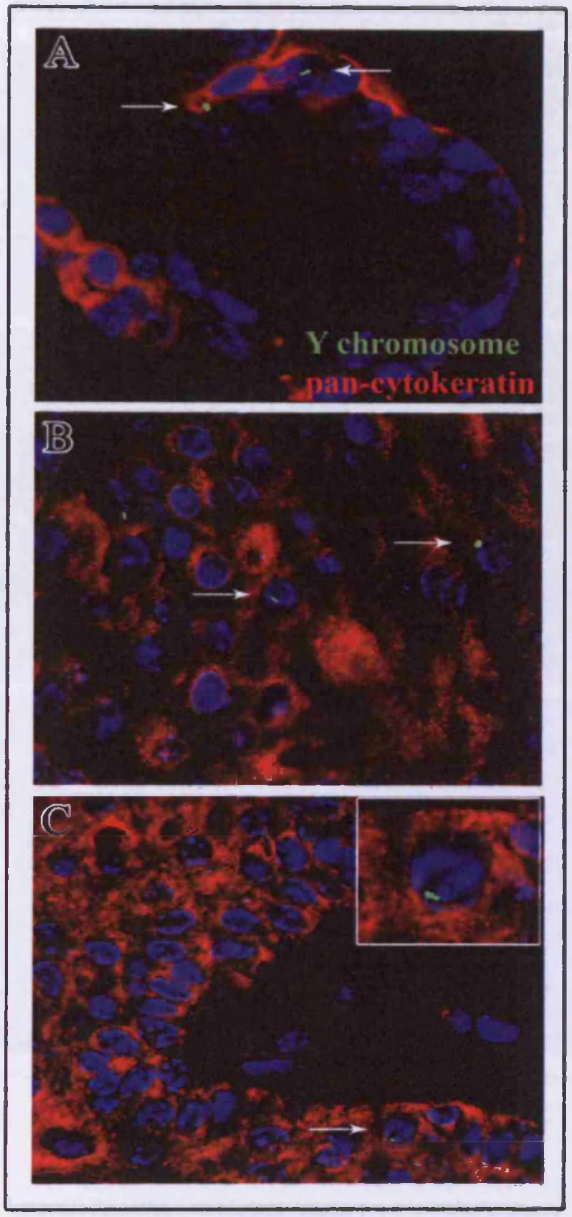


Figure 6.5. Donor derived cells of epithelial nature as shown by combined immunohistochemistry for pan-cytokeratin and Y-FISH. Cells engraft as epithelial cells in a sporadic fashion (A) normal epidermis, (B,C) skin tumours. Epithelial donor-derived cells are marked by arrows. Cell indicated by arrow in (C) is magnified in inset.

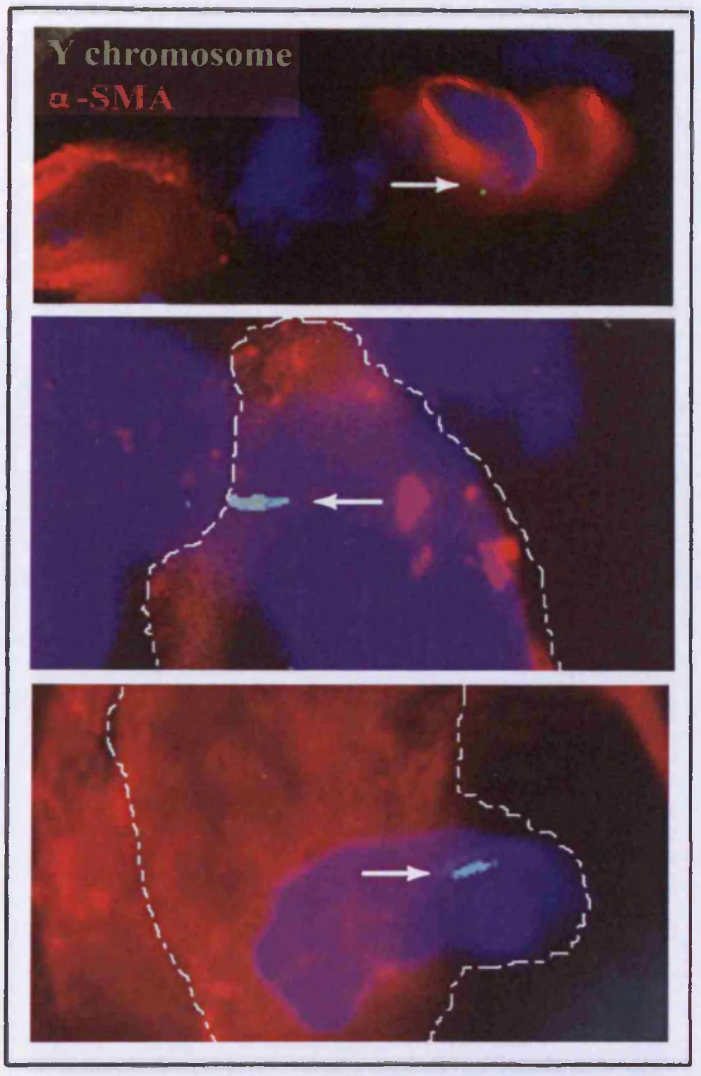


Figure 6.6. Donor-derived cells engrafted into the tumour stroma as shown by expression of α-SMA (red) and presence of the Y-chromosome (green). Outlines of cell borders are marked. Arrowheads indicate donor-derived myofibroblasts expressing α-SMA.

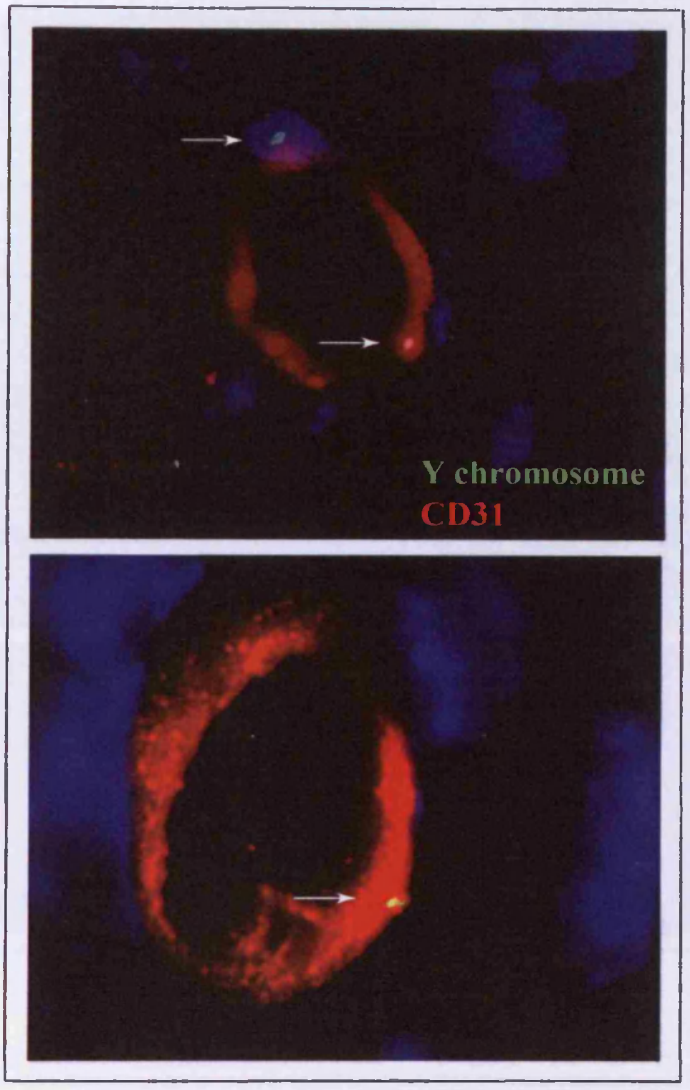


Figure 6.7. Donor derived endothelial cells as shown by combined immunohistochemistry for CD31 and Y-FISH. Cells engraft as endothelial cells in a sporadic fashion. Endothelial donor-derived cells are indicated by arrows.

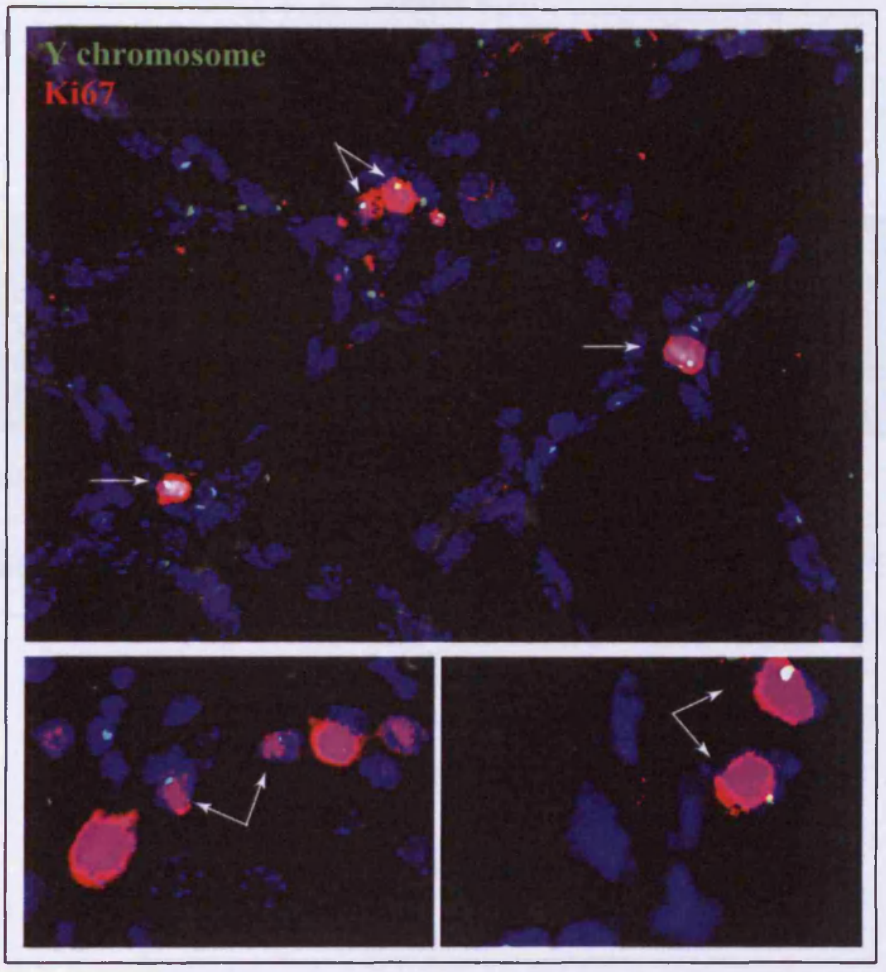


Figure 6.8. Donor derived cells that are actively dividing as shown by combined immunohistochemistry for Ki-67 and Y-FISH. Proliferating donor-derived cells occur throughout the entire sections and are marked by arrows.

Table 6.1

Tumour development 20 weeks after BM transplantation

Recipient / BM	HPV		wt	
	Wounding	No wounding	wounding	No wounding
HPV	3/3	3/3	0/3	0/3
wt	3/3	3/3	0/3	0/3

CHAPTER VII

CLONALITY IN SKIN TUMOURS AND IMPLICATIONS FOR LINEAGE RELATIONSHIPS IN THE SKIN AND THE ORIGINS OF BASAL CELL CARCINOMAS

7.1 INTRODUCTION

7.1.1 The human skin

The skin is the largest organ in the human body. Its multilayered epthelium comprises the interfollicular epidermis (IFE), interspersed with hair follicles, sebaceous and sweat glands. The cells of the IFE and sebaceous glands follow a single terminal differentiation pathway to form the stratified cornified epithelial layer and the lipid filled sebocytes, respectively. Hair follicles comprise eight different cell lineages. Medullary cells lie in the centre, surrounded by cortical and hair cuticle cells, which are in turn surrounded by the three cell lineages of the inner root sheath (IRS). This is covered by the outer root sheath, which lies upon a companion layer separating the IRS and ORS (reviewed in (Watt 2002, Watt *et al.* 2006).

Cells are continuously shed from the epidermal surface and homeostasis is maintained by basally situated self-renewing stem cells (SC). Several distinct SC compartments have been proposed to replenish the various lineages (Ghazizadeh *et al.* 2001). However, cells from these different SC pools seem to retain multipotential characteristics when exposed to the appropriate stimuli and can be triggered to interconvert, as described in more detail in chapter I (Watt 1998, Panteleyev *et al.* 2001, Taylor *et al.* 2000, Oshima *et al.* 2001, Reynolds *et al.* 1992, Ferraris *et al.* 1997, Miller *et al.* 1998). It appears as though under normal conditions, SCs follow their normal pattern of differentiation, retaining multilineage potential for emergency situations, such as injury or stress.

The IFE SCs are believed to be organised in small clusters of basal quiescent cells surrounded by proliferative transit amplifying cells (TAC), which after a few cell divisions undergo terminal differentiation (Jones *et al.* 1995, Jensen *et al.* 1999). Controversy exists about the number of SC populations in the hair follicle.

A widely accepted SC pool, claimed by some to be the the only one, is located in the hair bulge, a specialised region in the ORS (Fuchs *et al.* 2001). According to the Unitarian hypothesis, bulge SCs form the ORS and additionally move along the ORS to the matrix of the hair bulb, where they function as SCs to replenish IRS and hair shaft (Kopan *et al.* 2002). Another theory, backed up by lineage tracing studies, claims that the cells generating the IRS lineages reside in a separate stem cell pool in the matrix at the base of the hair follicle, whereas the ORS is derived from bulge SCs (Kamimura *et al.* 1997, Blanpain *et al.* 2004, Legue *et al.* 2005). However, it is unclear whether the matrix progenitor cell represents a SC itself, thus separating the developmental origins of IRS and ORS, or whether it is merely a progenitor pool, fed from bulge SCs. The evolutionary origin of the companion layer and its attribution to either the IRS or the ORS is also subject to debate. Analysis of keratin expression patterns ascribed it to the IRS as its outermost layer (Gu *et al.* 2007).

Much research has focused on identifying markers of skin stem cells. Putative markers include Wnt-inhibiting factor (Wif1) and Dickkopf (Dkk3) (reviewed in (Watt *et al.* 2006)), the bulge-specific keratin 15, α6 and β1 integrins, and as recently suggested, CD200 (Terunuma *et al.* 2007). CD34 has also been explored as skin SC marker, due to its expression in the ORS of anagen follicles just below the 'bulge', a known SC zone in the hair follicle (Poblet *et al.* 1994).

7.1.2 Skin tumours and their origins

Constant exposure to environmental assaults, such as UV radiation, chemicals and pollution makes the skin vulnerable to carciogenesis. Different types of epidermal tumours show variances in cell lineage differentiation, arguably reflecting the SC pool they originated from. For example, SCCs have elements of IFE differentiation and are thought to originate from the IFE SC pool. Trichofolliculomas and pilomatrichomas show differentiation towards the hair shaft and are thus believed to originate in the SC pool that gives rise to these lineages.

BCCs are the most common skin tumours in humans. Although they can be invasive tumours, they do not metastasise. BCCs display a homogenous appearance and it is conceivable that they arise from the same SC pool as the similar looking ORS. This relationship is supported by the fact that sonic hedgehog (SHH) signalling, the malfunction of which results in BCC development, only occurs in hair follicles.

There is evidence for a cellular hierarchy within skin tumours (reviewed in (Perez-Losada *et al.* 2003)). The now classical experiment if chemical tumour initiation and promotion showed that the only cells with a sufficient lifespan to have been present at the time of initiation are SCs (Van Duuren *et al.* 1975). In fact, SCs seem to be necessary for tumour development, since CD34 knockout mice fail to develop papillomas in initiation-promotion experiments (Trempus *et al.* 2007). The stem cells of the hair bulge have been shown as a target for UV-induced mutagenesis and the source of SCCs, since mice that had their IFE removed, still developed SCCs in response to UVB radiation (Faurschou *et al.* 2007).

Skin tumours are believed to be clonal lesions. Mutation tracing studies have shown differences in clonality patterns between different types of skin tumours; in particular they have revealed a link between actinic keratoses and SCCs, but not with BCCs. Despite a morphologically multifocal appearance, genetic analysis, such as X chromosome inactivation and loss

of heterozygosity (LOH) of chromosome 9 (9q22.3), and three-dimensional reconstructions of BCCs have favoured a unicellular origin (Walsh *et al.* 1996, Shulman *et al.* 2006). However, in some tumours, tracing molecular markers suggested polyclonality. This could indicate an independent origin of two or more tumours, or show subclonality following a late mutation in carcinogenesis (Asplund *et al.* 2005). SCCs are believed to harbour more genetic heterogeneity. Comparison of the LOH patterns in oesophageal SCCs has suggested that they are clonal neoplasms with a considerable degree of divergence and genetic progression (Shima *et al.* 2006)

Controversy exists regarding the origin of the stroma associated with BCCs. Xenograft studies have suggested that BCCs generate their own 'specialised stroma', interspersed with cells derived from the host, whereas other researchers believe that BCCs are embedded in a connective tissue stroma, at least in part of polyclonal origin (Stamp *et al.* 1988, Hales *et al.* 1989, Asplund *et al.* 2005).

7.2 AIMS

There is much debate concerning the number of SC pools in the human skin and the dynamics and origins of skin tumour development. Following the spread of mt mutations in a variety of human skin tumours, we aimed to provide insight into the origins and dynamics of skin cancer development and additionally to shed light on the dispute regarding lineage relationships in the skin. To do so, we applied the techniques well established and widely used in our laboratory in the study of clonality and spread of mutations in the gastrointestinal tract to the study of the epidermis. This may provide a better understanding into the development, risk factors and therapy of skin cancer.

7.3 MATERIALS AND METHODS

7.3.1 Patients

For this study, six patients that underwent surgical resection of skin tumours were selected. Specimens were snap-frozen in liquid nitrogen-cooled isopentane or fixed in 4% PFA and embedded in paraffin. Ethical approval was sought and obtained as per the requirements of the United Kingdom Human Tissue Act (2006), REC reference number 06/Q0603/1.

7.3.2 Immunohistochemistry

Paraffin-embedded sections were de-waxed and blocked, frozen sections were dried and fixed in cold acetone. After appropriate antigen retrieval for paraffin-embedded sections, non-specific staining was blocked by incubation in rabbit serum for 25 min. Sections were successively incubated for 35 min with anti-cytochrome c oxidase subunit 2, rabbit anti-mouse IgG and Strep-HRP. Antibody staining was detected with DAB and sections were counterstained with haematoxylin.

7.3.3 Cytochrome c oxidase enzyme histochemistry

Frozen sections underwent successive histochemical staining at 37°C for cytochrome c oxidase and succinate dehydrogenase, the detection of the latter indicates absence of cytochrome c oxidase activity. Incubation in cytochrome c oxidase was performed until enzyme activity was optically detectable by brown staining. This was followed by an incubation in SDH incubation medium to detect succinate dehydrogenase until a blue colour was detectable.

7.3.4 Laser capture microdissection

Cells from cytochrome c oxidase-positive and negative areas were cut by laser microdissection. Cell digestion and DNA extraction was performed by overnight incubation in a DNA extraction kit (Picopure™) at 65°C.

7.3.5 PCR and Sequencing

The extracted DNA was used to sequence the entire mitochondrial genome from microdissected areas. A two-round amplification method was followed, whereby the first round consisted of amplifying 9 fragments spanning the entire genome, and the second round consisted of 36 M13-tailed primer pairs to amplify overlapping segments of the first-round products. Sequencing was performed using the BigDye terminator cycle sequencing method on an ABI Prism Genetic Analyzer and compared to the revised Cambridge reference sequence using sequence alignment software of the European Molecular Biology Open Software Suite (EMBOSS).

7.4 RESULTS

7.4.1 Different types of skin tumours vary in clonality patterns

Cytochrome c oxidase enzyme activity and immunohistochemistry showed distinct patterns of cytochrome-deficient cells in different tumour types. SCCs displayed small and frequent patches of cytochrome oxidase-deficiency dispersed throughout the lesions. The cytochrome-deficient areas mostly comprised the cornified islands, and appeared as though they are fed from the immediate surrounding squamous cells that show distinct heterogeneity in cytochrome oxidase deficiency (Figure 7.1, og228). In BCCs, we observed a more homogeneous distribution of cytochrome oxidase deficiency (Figure 7.2A-E, pg.229). In most cases, the cytochrome oxidase-deficiency included the entire lesion, which interestingly was also observed to include the tumour stroma (Figure 7.3, pg.230). In one case we observed a homogeneous cytochrome-deficiency throughout the entire lesion that extended to a histologically normal ORS of a hair follicle, suggesting a developmental link (Figure 7.2 D,E, pg.229). However, some BCC sections also showed patches of cytochrome deficient areas dispersed throughout the lesions, albeit they were larger areas than the patches observed in SCCs.

7.4.2 Cytochrome c oxidase-deficient areas share identical mutations

Comparison of the mitochondrial sequences of cells from various tumour areas to the revised Cambridge Reference Sequence (rCRS) revealed sequence variations throughout the entire mitochondrial genome. Similar to the observations in the gastrointestinal tract, we detected on average approximately 1-2 mutations per amplified DNA segment (app 400 base pairs). The detected sequence divergences included common polymorphisms published in the human mitochondrial genome database (Mitomap), as well as novel mutations.

Sequence comparison of mt DNA extracted from blue and brown areas of the tumours showed a high sequence homology. Most mutations were shared by various cytochrome oxidase-deficient and cytochrome oxidase-competent areas. However, a few mutations were identified in cytochrome oxidase-deficient areas, where neighbouring cytochrome c oxidase-positive areas displayed the wt sequence. Notably, in all tested tumours, the sequence divergence was only observed between blue and brown areas, but we never detected sequence differences between only the blue or brown areas, respectively. Some of the mutations shared by cytochrome-deficient areas occurred in the genes coding for cytochrome oxidase subunits, such as the synonymous 7415 A>G mutation in cytochrome c oxidase subunit I in the squamous tumour (carcinoma *in situ*) shown in Figure 7.4 (pg.231). However, sequence divergences between cytochrome oxidase-deficient and cytochrome oxidase-competent areas did not necessarily occur in cytochrome c oxidase subunit genes. We also observed other synonymous changes, such as 13740 T>C (Figure 7.5A, pg.232). Moreover, mutations were also found in other mt genes, such as the 3313 A>C and 3363 C>A in the NADH dehydrogenase subunit 1 (Figure 7.5B, pg.232), or the 4916 A>G transition seen in NADH dehydrogenase subunit 2 (Figure 7.4, pg 231). Although these changes most probably were not the cause of the deficiency in cytochrome oxidase, they show that a single mutation can be shared by various areas of the

tumours and even persist throughout the entire tumour and possibly originate in histologically normal tissue areas.

7.4.3 BCCs originate from normal SCs of the ORS

One of the BCC lesions in our study showed a pattern of cytochrome c oxidase deficiency that not only comprised all tumour buds of the lesion, but also extended to a histologically normal hair follicle ORS. Mutation analysis revealed 2 mutations shared by all examined blue areas of the ORS, as well as by tumour buds from spatially distinct locations of the tumour, whereas adjacent brown areas showed wt sequence (Figure 7.5, pg.232). This indicates a common origin of the normal ORS and the BCC lesion.

7.4.4 Companion layer and ORS originate from different SC pools

It is an open debate whether the companion layer separating the IRS from the ORS evolutionary belongs to the IRS or the ORS. Here, we observed cytochrome oxidase deficiency throughout the outermost layer of the ORS, but layers beneath, including the companion layer were cytochrome oxidase-competent, providing evidence that the companion layer is not rooted in the same SC population as the ORS (Figure 7.2D,E).

7.4.5 Deficiency in cytochrome c oxidase does not affect cell function

To investigate whether the mutations that were traced in order to investigate tumour development confer a pathological effect on cell activity, cell functionality of the epithelial tumour cells was tested by expression of cytokeratin. Using a pan-cytokeratin antibody, we confirmed that all cells within the cancer are functional epithelial cells, irrespective of their deficiency of cytochrome oxidase. Patches that showed a deficiency in cytochome c oxidase activity upon the cytochrome c oxidase enzyme reaction, stained for pan-cytokeratin with the same level of intensity as observed in the cytochome oxidase-active patches (Figure 7.6, pg.233).

7.5 DISCUSSION

7.5.1 Clonality and multiple cancer SCs in skin tumours

In this study, we observed distinct patterns of cytochrome c oxidase deficiency in different types of skin tumours, indicating that different tumour types differ in their developmental dynamics and origins. Dispersed patches of cytochrome oxidase-deficiency were observed in all examined SCCs. This intratumour heterogeneity could either indicate that SCCs generally have a high mutation rate during tumorigenesis, or that SCCs are of polyclonal origin and harbour multiple stem cells. However, the limited number of mt mutations and consistency thereof in all cytochrome oxidase-negative areas suggests that the number of founding cancer stem cells is limited and that cytochrome-deficient areas might be related on a three-dimensional level. It is also conceivable that the original initiating SC harboured a considerable level of heteroplasmy that was passed on in an imbalanced way to its progeny, resulting in a falsely perceived polyclonality. However, this possibility would not be recreated at such consistency in all SCC samples, as observed in this study.

In BCCs, cytochrome c deficiency showed a much more homogeneous pattern. In the majority of tumours, the absence of cytochrome c oxidase extended throughout the entire lesions, suggesting a monoclonal origin. This uniformity was also confirmed on a molecular level, by detecting the same mutations in all areas of the multifocal tumours. A monoclonal origin could be challenged by the observation of cytochrome-deficient patches in some BCCs. Possible explanations of this phenomenon are the acquisition of mutations at later stages of tumour development, creating subclonal populations. It was previously suggested, that the perceived polyclonality could also reflect the simultaneous presence of more than one distinct tumour. The genetic heterogeneity could be based on heteroplasmy within the founding CSC. This is more conceivable than in SCC lesions as mentioned above, due to bigger patch sizes and the fact that most BCCs seem to be of monoclonal origin both, in our studies, as well as in the literature. Interestingly, cytochrome oxidase-deficiency in our study

generally also included the stroma surrounding the BCC lesions. Although a common origin has not been molecularly confirmed yet, the consistency of the results obtained in all other tumour areas indicate that BCCs and their stroma are derived from a common ancestor. This confirms previous claims based on xenograft studies that BCCs generate their own 'specialised' tumour stroma (Stamp *et al.* 1988), rather than recruiting a polyclonal stroma from the host as seems to be the case with most cancers.

Taken together, these results suggest that BCCs and SCCs follow distinct developmental pathways originating in separate SC pools, which is also reflected by tumour-specific differences in cell lineage differentiation. Accordingly, this suggests that BCCs are monoclonal lesions, whereas SCCs seem to be of polyclonal origins, stemming from several SCs with no sign of monoclonal conversion.

7.5.2 Monoclonality of BCC and its origin in hair bulge SCs

The pattern of cytochrome oxidase deficiency with its identical mutation profile across various tumour locations suggests that BCCs are typically monoclonal, originating from a single pool of CSCs. This has been suggested before and, based on histological observations, researchers have suggested that the origin of BCCs may lay in the SCs of the hair follicle bulge. However, this hypothesis has never been experimentally confirmed. In this study, we detected a BCC that showed a distinct pattern of cytochrome oxidase-deficiency, displaying a continuity extending from a histologically normal ORS of a hair follicle to the entire tumour. The common origin of this monoclonal phenomenon was confirmed by mutation analysis of various cytochrome oxidase-deficient areas. These findings provide evidence for a common origin of BCCs and the ORS in the SCs of the hair bulge. Interestingly, the ORS is histologically normal and the neoplastic phenotype appears to only affect the interfollicular areas. This might be due to a different rate of cell division between the two different

tissue areas, or local microenvironmental cues that keep the cells in the ORS in check.

7.5.3 The origin of the companion layer

Patterns of cytochrome c oxidase activity and molecular analysis confirm that the ORS is founded in a SC pool distinct from the IRS and the hair shaft. The origin of the companion layer has been a topic of discussion and has been attributed to both, the SCs in the hair bulge thus presenting a lineage relationship with the ORS, as well as the hair shaft, rendering it evolutionary related to the IRS. Our results show that the companion layer is part of the cytochrome c oxidase-competent component of the tissue, thus attributing it evolutionary to the hair shaft as the outermost layer of the IRS.

7.6 CONCLUSION

In this study, we are contributing important information to the ongoing debate concerning lineage relationship status in the skin, as well as to the origin and development of different types of skin cancer.

We show on a histochemical and molecular level that different SCCs and BCCs are evolutionary distinct and develop from different SC pools. This study reveals several important aspects of lineage relationship in the skin and the development of skin tumours. We show that (1) BCCs are typically monoclonal and thus derived from one founding SC, whereas SCCs appear to be polyclonal as evidenced by their multiple dispersed patches of cytochrome oxidase c deficiency throughout the tumours. (2) BCCs and SCCs most probably differ in their SC type of origin, which is also reflected in their histological characteristics (3) ORS and BCCs originate from the same SC pool, presumably in the bulge of the hair follicle. (4) The SC pool generating the ORS and BCCs is distinct from the one generating IRS lineages. (5) The companion layer is evolutionary distinct from the ORS and thus part of the IRS and can thus be viewed as its outermost layer.

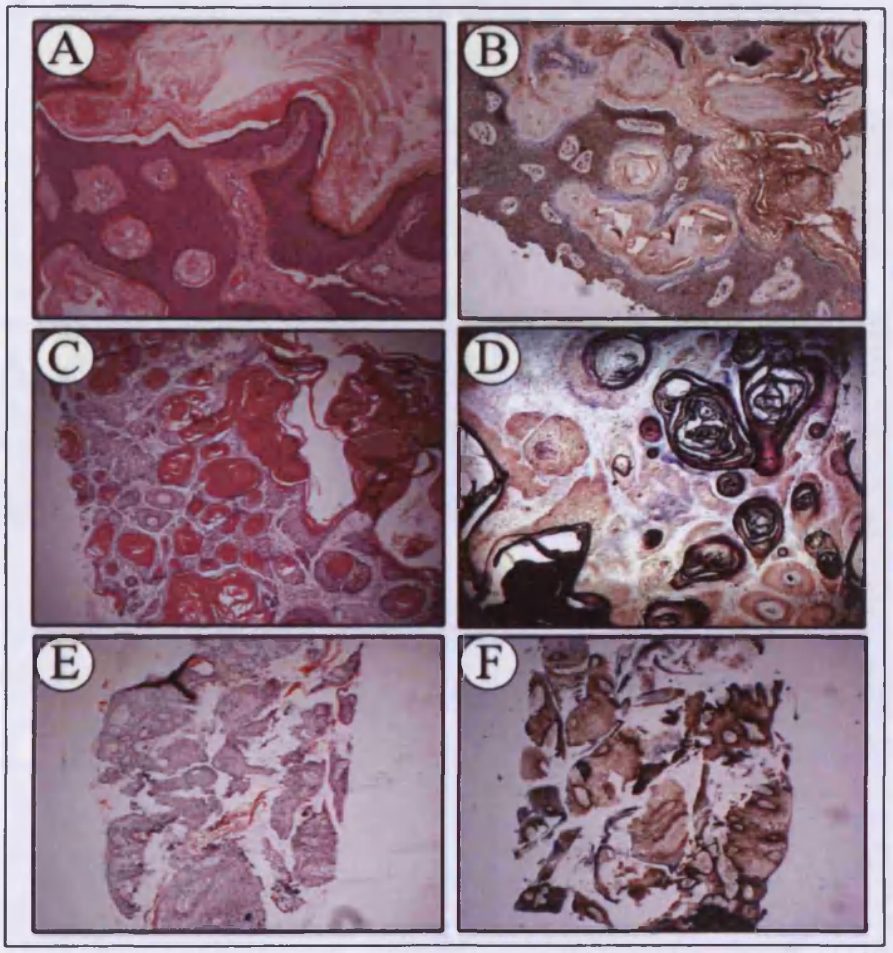


Figure 7.1. Typical patterns of cytochrome c oxidase-deficient patches in squamous tumours. (A,C,E) H&E staining identifying sections (A,C) as squamous cell carcinomas and section (E) as carcinoma *in situ*. (B) Immunohistochemistry for cytochorome c oxidase expression on serial section of the tumour shown in (A) showing patches lacking cytochrome c oxidase (blue) dispersed throughout the lesion. (D,F) Cytochrome c oxidase histochemistry on the biopsy shown in (C,E), displaying patches lacking cyto chrome c oxidase enzyme-activity (blue) and patches with active cytochrome c oxidase enzyme (brown) in the same tumour

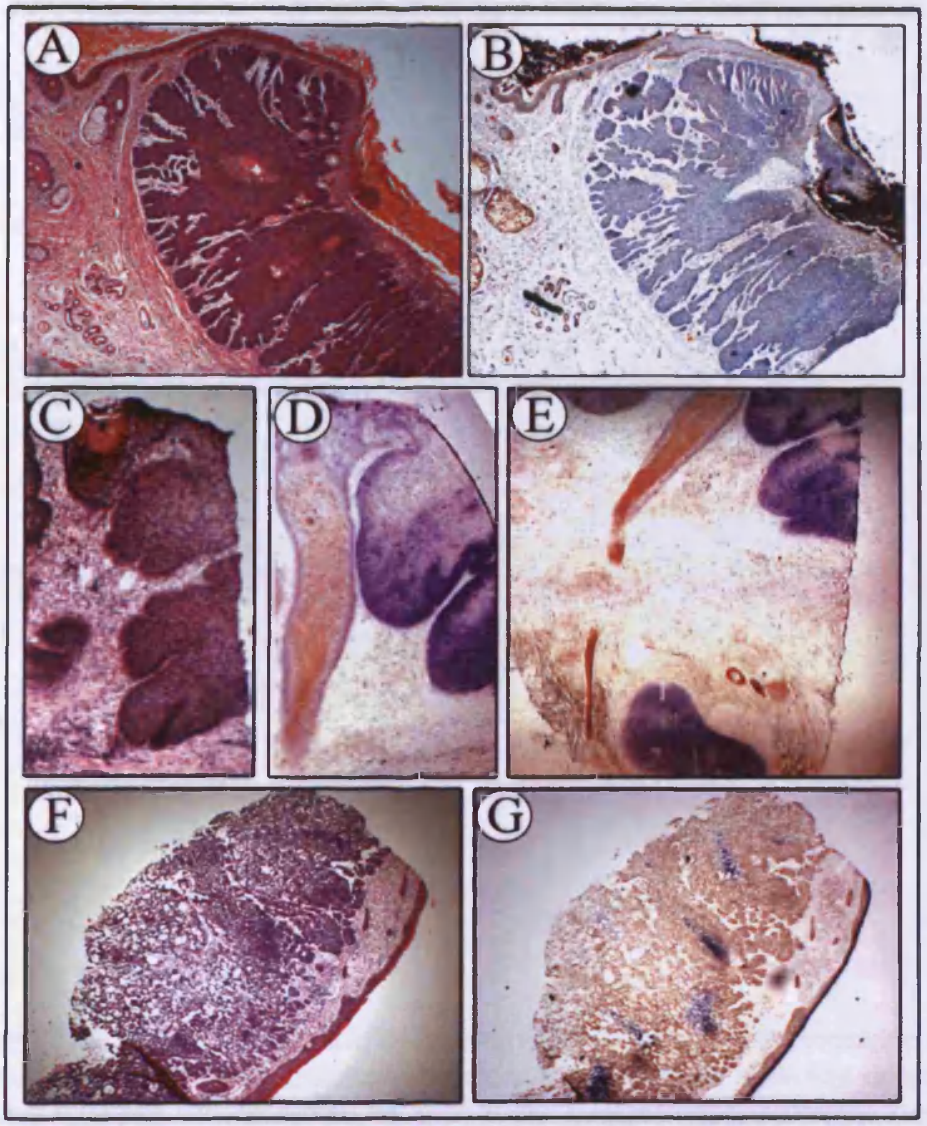


Figure 7.2. Cytochrome c oxidase-deficiency in BCCs. (A,C,F) H&E staining identifies lesions as BCCs. (B) Immunohistochemistry for cytochrome oxidase expression shows that the entire tunour is homogenously cytochrome oxidase-negative (blue), whereas normal epithelium and surrounding tissue expresses the protein (brown). (D,E) Cytochrome c oxidase enzyme activity assay performed on serial sections of lesion shown in (C), displaying cytochrome c oxidase deficiency throughout the tumour buds, extending to histologically normal ORS of a hair shaft (blue). The inner cell lineages of the hair shaft show activity of cytochrome c oxidase (brown). (G) Cytochrome c oxidase enzyme activity assay performed on serial section of lesion shown in (F) showing patches of cytochrome c deficiency amidst a largely cytochrome c oxidase-competent tumour.

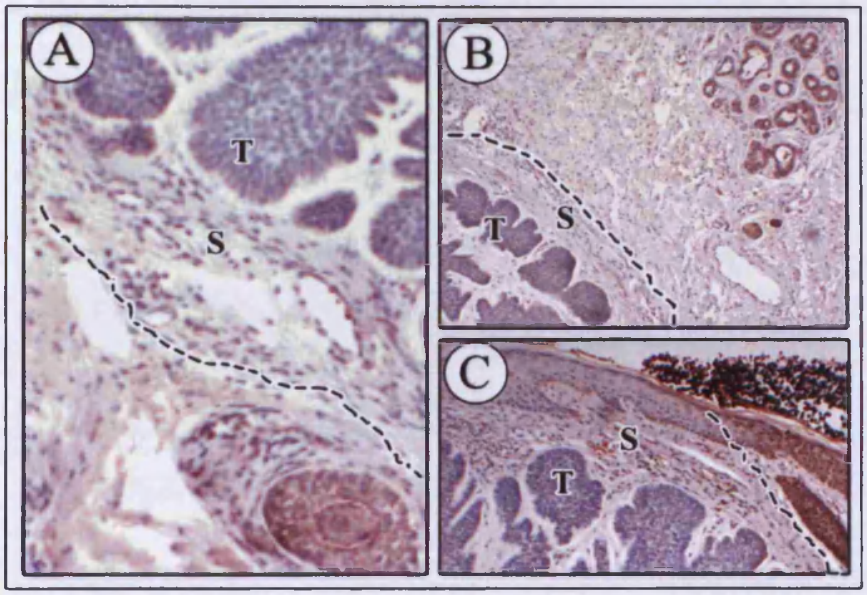


Figure 7.3. A BCCs including its surrounding stroma and theoverlying epidermis are devoid of cytochrome c oxidase expression, whereas adjacent tissues express the enzyme, as shown by immunohistochemistry. Dotted lines outline approximate boundary of cytochrome c oxidase expression. (T=Tumour, S=Stroma).

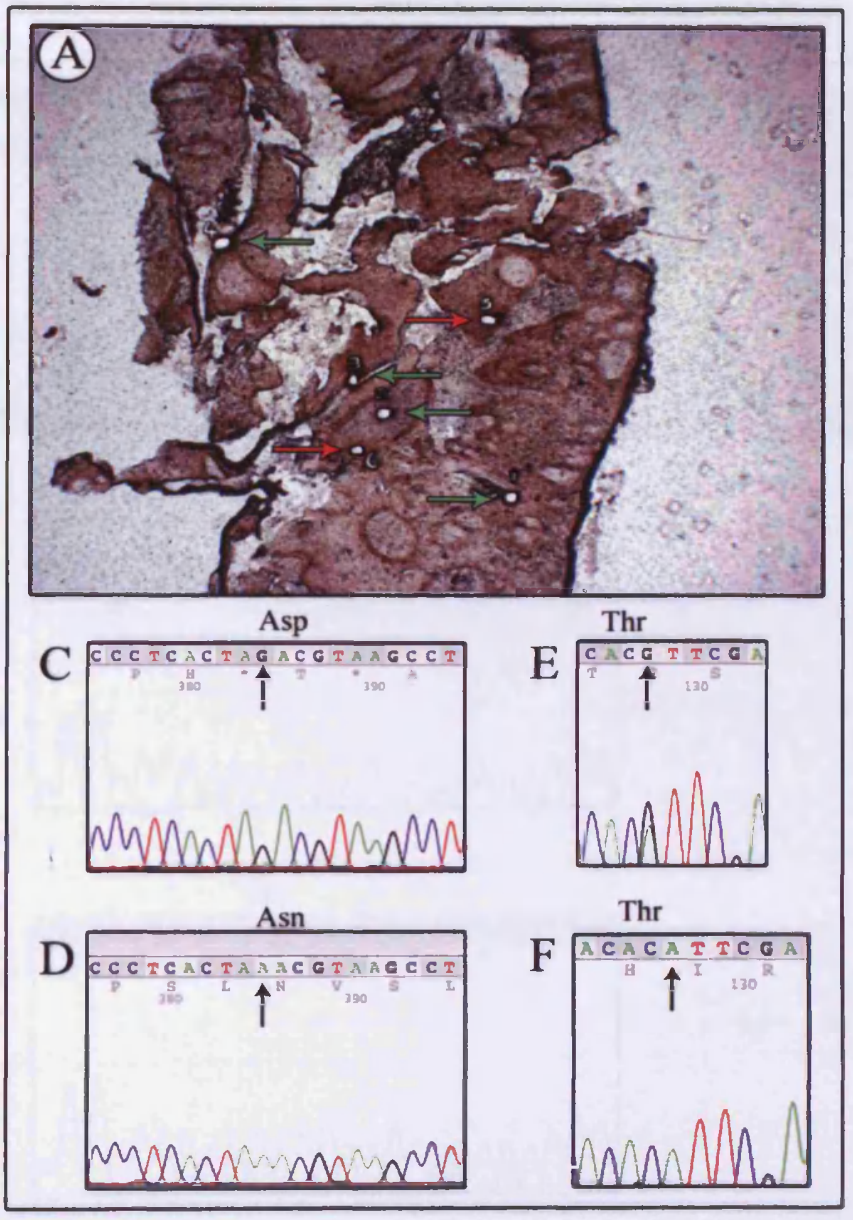


Figure 7.4. Sequence analysis of DNA from microdissected cells in cytochrome c oxidase-deficient (blue, indicated by green arrows) and competent (brown, indicated by red arrows) areas of the squamous tumour shown in (A). (C,E) Sequence mutations shared by all blue areas, showing a 4916 A>G and 7415 A>G mutation (arrows). (D, F) The brown areas show the wt sequence (arrows).

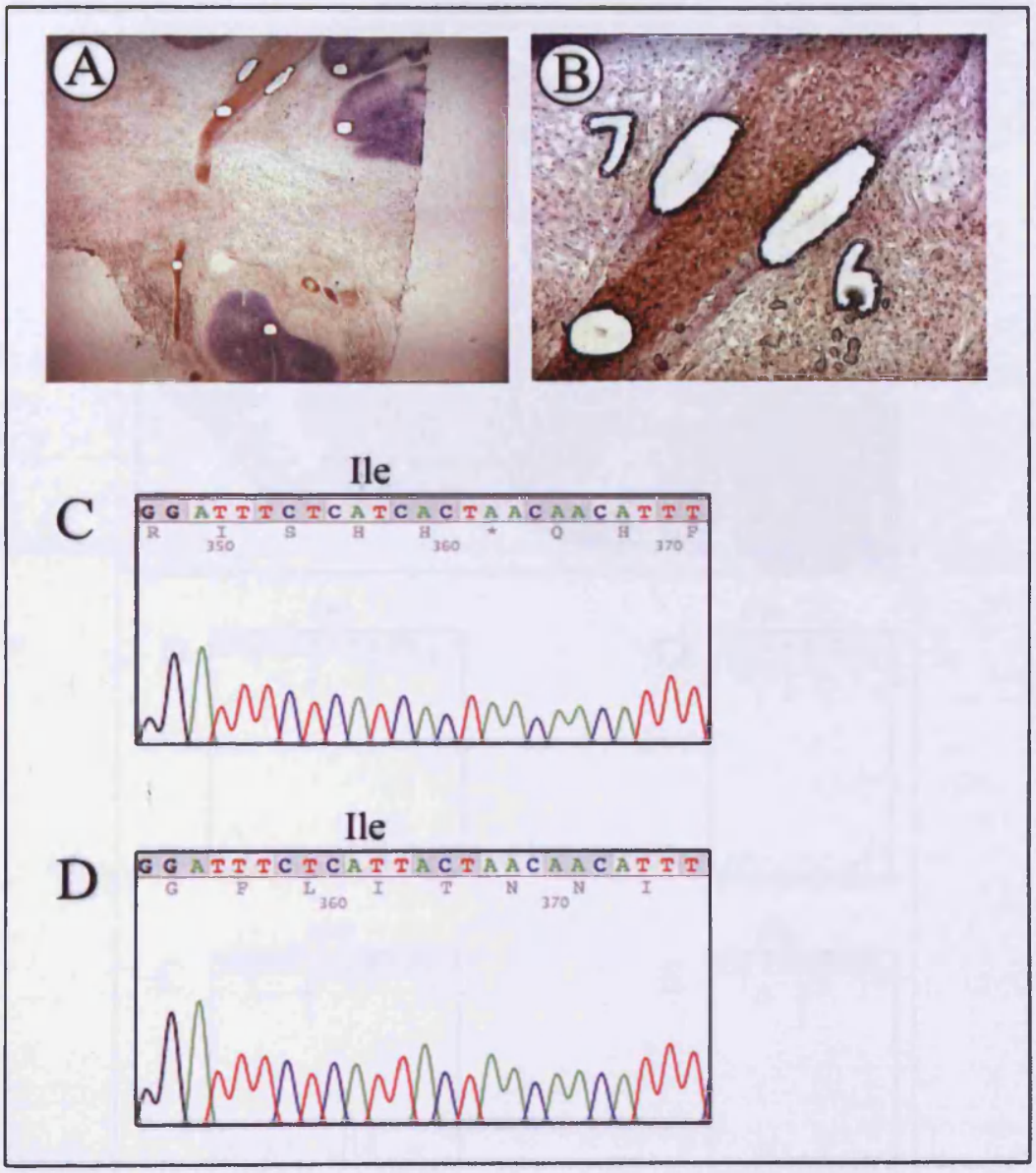


Figure 7.5a. Sequence analysis of DNA from microdissected cells in cytochrome c oxidase-deficient (blue) and competent (brown) areas of the BCCshown in (A) and (B). (C) All blue areas share a 13740 T>C mutation, whereas (D) the brown areas show the wt sequence.

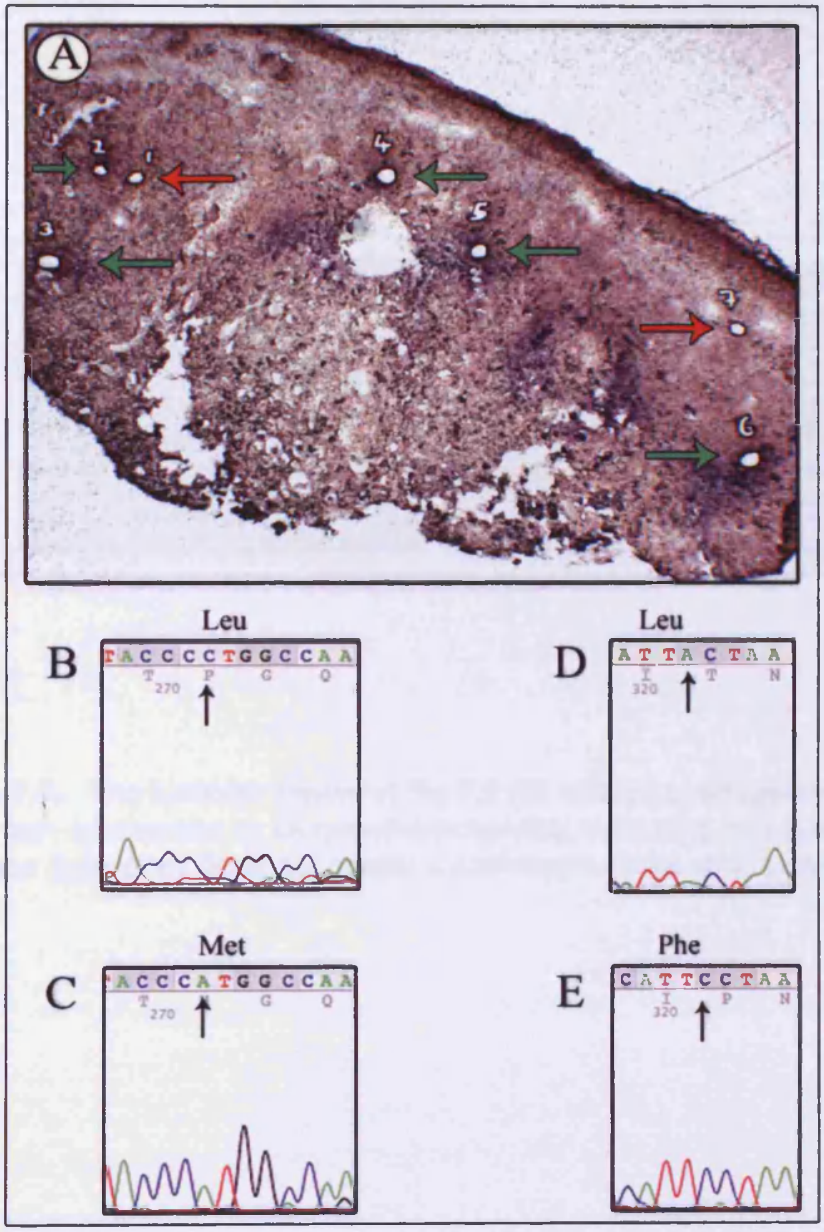


Figure 7.5b. Sequence analysis of DNA from microdissected cells in cytochrome c oxidase-deficient (blue, indicated by green arrows) and cytochrome oxidase competent (brown, indicated by red arrows) areas of the BCC shown in (A). (B,D) All blue areas show mutations 3313A>C and 3363C>A. (C,E) The brown areas show the wt sequence in both locations.

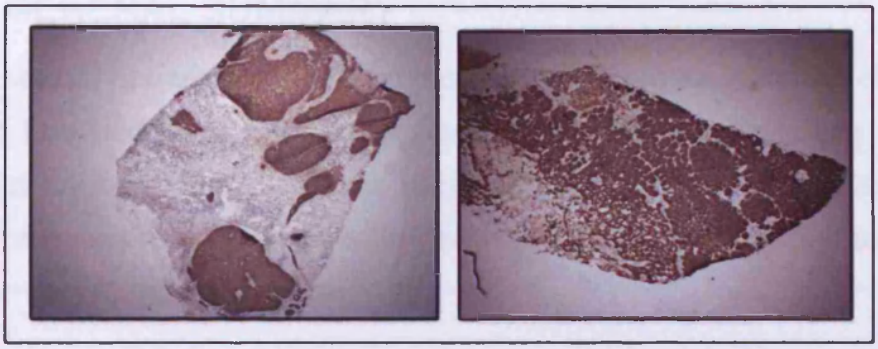


Figure 7.6. The tumours shown in Fig 7.2 (C) and (F) stain uniformly positive for pan-cytokeratin by immunohistochemitry, indicating that cytochrome c oxidase deficiency does not confer a pathologic effect on the cells..

CHAPTER VIII

DISCUSSION

8.1 GENERAL SUMMARY

The research carried out in my project was aimed to expand and elaborate on the knowledge of cancer stem cells and cancer-originating cells in epithelial cancers, including their phenotype, their developmental dynamics and the reciprocal relationship between individual cells and their microenvironment.

Examination of cancer stem cells first focused on the applicability of the SP phenotype for isolation and identification of cancer stem cells in colorectal cancer cell lines. Our results showed that non-SP cells were identical in phenotypical and behavioural stem cell-like characteristics, defined by the expression of putative stem cell markers, clonogenicity *in vitro*, tumorigenicity *in vivo* and multilineage differentiation potential *in vitro* and *in vivo*. Based on these observations, the SP did not represent a stem cell-enriched population in colorectal cancer cell lines. Subseqently, we tested whether the combination of two surface markers, CD44 and CD133, that recently have been suggested as markers for stem cell-enriched populations, might characterise a stem cell-enriched population in primary human colonic adenocarcinomas. Tumours showed an upregulation of both surface markers and non-random distribution restricted to the columnar cells facing the luminal surface of the gland-like structures typically found in colonic adenocarcinomas. However, we could not confirm stem cell characteristics of isolated double-positive cells *in vitro* by sphere formation assays or *in vivo* by subcutaneous injections of double-positive cells into nude mice.

Two projects were carried out to investigate the effect of cells originating in organ or BM transplantations on the host in particular with respect to their potential to act as cancer-originating cells in the skin. First, post-transplant skin tumours of human female kidney transplant patients who had received a male organ were tested for Y-positive genetic material in a large-scale study, involving 35 patients and 176 skin tumours.

Second, a study was carried out involving a mouse model of spontaneous skin carcinogenesis to test the applicability of the above-mentioned results to mouse models and take advantage of the possibility to control the experimental settings. The effects of genetic predisposition of transplanted cells and the local microenvironment on each other was tested by reciprocal BM transplantations involving wt C57/Bl 6 mice and an HPV8 transgenic mouse model of spontaneous skin carcinogenesis. Our findings were similar to the observations made by other researchers, namely, that donor-derived cells engraft in the human normal epidermis and skin tumours sporadically without evidence for cell fusion or stem-cell typical characteristics, such as clone formation or preference for known stem cell niches (Hemmatti *et al.* 2003, Cogle *et al.* 2007). Monitoring tumour development and cellular make-up revealed that the phenotypic outcome was largely dependent on the host genetic make-up and was not significantly altered by the engraftment of transplanted cells.

We thus reach the conclusion that in the absence of chronic injury/inflammation such as described by Houghton *et al.* (2004), donor-derived cells contribute to the tissue by adjusting their phenotype and thus mimicking the cells in their surroundings rather than playing a dynamic active role in carcinogenesis and tissue homeostasis. We propose a scenario, whereby circulating donor-derived leukocytes that were transferred with the grafted organ may be capable of transdifferentiating into epidermal and stromal cells when exposed to the appropriate biochemical stimuli. It appears as though the type of transplantation plays a role in the engraftment process. BMT generally results in a higher level of engraftment, plasticity and the occurrence of BM-derived tumours. The

donor-origin of the only donor-derived tumour after a solid organ transplantation could not be completely validated, and could be stemming from an earlier blood transfusion or an extremely rare phenomenon.

Furthermore, this research contributes new information to the development dynamics and origins of BCCs and to the ongoing debate concerning lineage relationships in the skin. Using mt DNA mutations as a marker of clonality, I show that BCCs and SCCs most probably differ in their SC type of origin, which is also reflected in their histological characteristics. Whereas I found a polyclonal appearance in SCCs, BCCs were histologically and genetically monoclonal and thus derived from one founding CSC. Molecular continuity with a histologically normal ORS suggests that both the ORS and BCCs originate from the same SC pool, presumably in the bulge of the hair follicle. I also provide evidence for an evolutionary distinctness between the ORS and IRS lineages and the evolutionary relationship of the companion layer with IRS, whereupon it can be viewed as its outermost layer.

8.2 PHENOTYPICAL CHARACTERISATION OF CANCER STEM CELLS

Following the discovery of the SP phenotype and its initial presentation as a stem cell-enriched population, a surge of research has emerged claiming stem cell characteristics in SPs from a wide variety of murine and human cell lines, normal tissues and tumours (Srour *et al.* 1993, Asakura *et al.* 2002, Alvi *et al.* 2003, Bhattacharya *et al.* 2003, Dekaney *et al.* 2005, Morita *et al.* 2006, Szotek *et al.* 2006, Chiba *et al.* 2006). Altogether, the evidence suggested that the SP may represent a universal stem cell marker. With the intention of finding a stem cell marker for the yet undefined stem cell population in normal and malignant gastrointestinal tissues, my research was undertaken to test the SP of gastrointestinal cancer cell lines for stem cell-typical phenotypes and behaviour. However

during the course of my experiments, increasing evidence has emerged challenging this notion (Mitsutake *et al.* 2007, Morita *et al.* 2006). Additionally, upon close examination many of the studies supporting the SP as a stem cell marker have neglected to directly compare the behavioural characteristics of SPs and non-SPs. Moreover, as a large proportion of this research was performed on murine tissues, evidence about the human counterparts is limited. Our research directly compared the SPs and non-SPs from various human gastrointestinal cancer cell lines with regard to phenotypical and behavioural stem cell-like characteristics. The two populations showed similar clonogenicity *in vitro*, formed xenografts *in vivo* with no differences in number, size, latency, or histopathology and were both multipotential with the ability to generate enterocytes, endocrine and mucus cells. Additionally, we showed that SPs and non-SPs are interchangeable; able to generate each other and reconstitute the original SP profile. Exposure to 5-FU, a lipophilic chemotherapeutic agent, did not selectively compromise any population, which would be expected for a population with a lower stem cell fraction. Moreover, 5-FU also did not alter the fractions expressing the putative stem cell surface markers CD133, CD44, CD117, and BCRP1, suggesting that these molecules are legitimate candidate markers for stem cells, since any change in expression profile would indicate a population's vulnerability to the toxic effects of the chemotherapeutic agent. The validity of our results was confirmed by the absence of a detrimental effect of Hoechst 33342 on cell viability.

The only difference we could detect between non-SP and SP cells in our experiments was the initial absence of CD34-expression on the SP fractions. In the literature, CD34-expression (Krause *et al.* 1994, Morel *et al.* 1996, Srour *et al.* 1993) as well as its absence (Cheng *et al.* 1996, Goodell *et al.* 1997) has been associated with stem cells in a tissue and species-specific manner. In our experiments, CD34-negative SP cells were able to generate CD34-positive cells in xenograft tumours, but this phenotype did not confer any behaviour that would indicate a stem cell-enriched population. A behavioural significance for this phenotypical phenomenon has yet to be elucidated. In agreement with recent reports

(Patrawala *et al.* 2005, Morita *et al.* 2006), we could not support a correlation between the SP phenotype and expression of BCRP1, indicating that other membrane transporters must play a role in conferring the ability to efflux Hoechst dye.

CD44 and CD133 have both been implicated as markers for stem cell enriched populations. In colorectal carcinomas, the CD44+/EpCAM^{high} population was shown to be tumorigenic when injected subcutaneously into NOD/SCID mice (Dalerba *et al.* 2007). A recent report indicated that CD133+ cells may also represent a stem cell-enriched population in colonic carcinomas. The CD133+ fraction of between 2.5-9% of all cells in primary human colorectal cancer cells was capable of sphere formation in serum-free medium and was 200-fold enriched in serially transplantable tumorigenic cells upon renal capsule or subcutaneous injection into immunodeficient mice (O'Brien *et al.* 2007, Ricci-Vitiani *et al.* 2007). I was able to demonstrate a higher expression of putative stem cell markers CD44 and CD133 in epithelial cells from adenocarcinomas of the human colon than in the normal colonic mucosa. The expression of CD44 was widely distributed throughout the tumours, whereas CD133 expression was restricted to the apical surface of cells lining the duct-like structures in the adenocarcinomas. The abundance of both markers suggests that they would not represent a pure stem cell population, but the combination of both surface markers only identified a few cells, which were distributed throughout the tumours but always occurred in the basal columnar cells lining the duct-like structures within the tumours. However, when testing stem cell-like behaviour, we were not able to replicate the results from the above-mentioned studies and additionally could not find similar stem cell-like behaviour when combining both surface markers. After one-week in culture in serum-free medium, no viable cells or spheres were detected and moreover, none of the isolated cell fractions resulted in xenograft formation in nude mice. These results may reflect technical difficulties and differences such as my choice of immunodeficient mouse model or my route of injection. Reduced cell viability after sorting was ruled out by testing viability before the *in vivo* injection and culturing of cells. Thus, the combination of

CD44 and CD133 may not be a suitable candidate marker combination to isolate and purify cancer stem cells in human colorectal adenocarcinomas.

Human cells were found in the matrigel pellets 4 months post-injection in small numbers and to varying degrees. Matrigel pellets injected with CD133+, CD44+ and double-positive fractions from normal colonic mucosa did not show any surviving human cells. Interestingly, in pellets injected with tumour cells, the numbers of human cells correlated with the expected stem cell enrichment of the cell fractions, with the least human cells in CD44-containing pellets and larger human cell clusters in pellets with double-positive cells. The detected human cells might well be progeny of the original injected cells since their longevity by far exceeds the normal lifespan of colonic epithelial cells and could potentially reflect the capability for self-renewal of a stem cell-enriched fraction. This could indicate that the CD133/44 double-positive cell fraction is most enriched in stem cells. However, small numbers did not allow for statistical evaluation of my observations, and considering the absence of operationally defined stem cell characteristics, such conclusions cannot be drawn. The results obtained by these experiments may also highlight the importance of the stem cell niche. It has previously been shown in our laboratory that single collagen embedded crypts can recruit host-derived stromal cells thereby initiating crypt and villus formation (Del Buono *et al.* 2005). These findings could not be extended to single cells. It is conceivable that the single-injected cells are not able to recruit a stem cell-supporting, host-derived matrix and thus cannot act upon their stem cell fate.

8.3 THE ROLE OF TRANSPLANTED CELLS IN TUMOUR DEVELOPMENT

Transplant medicine is faced with various secondary complications such as the development of post-transplant cancers due to immunosuppression, UV-irradiation and HPV-infection. In recent years, cells originating from the transplanted tissues have been repeatedly found in post-transplant malignancies, but their role in the development and the potential impact of the donor-genotype on the host system has yet to be elucidated.

In this thesis, we tested both, the extent and range of engraftment of donor-cells in post-transplant tumours in humans and the effect of genetically pre-disposed transplanted cells on post-transplant tumour formation in a mouse model.

8.3.1 Donor cell-engraftment in human post-transplant skin tumours

Close examination of the literature suggests that the degree of engraftment depends on the type of transplanted organ. Cells of haematopoietic origin appear to play a more significant role in the phenomenon of donor-cell engraftment both, regarding the diversity of plasticity and the amount of engraftment, as well as in the development of post-transplant tumours. For example, after BMT, 2-7% of cells in the normal skin were found to be donor-derived (Korbling *et al.* 2002, Hemmati *et al.* 2002), whereas after kidney transplantation, no donor-derived epidermal cells were found (Barozzi *et al.* 2003). Furthermore, post-transplant tumours that appear to be completely donor-derived are either found after BM transplantation or are of haematopoietic origin, such as Kaposi Sarcoma in kidney transplant recipients and leukemia in liver transplant recipients. These malignancies might stem from haematopoietic cells carried over with the transplanted organ. The only observation of solid tumour after solid organ transplantation, is a BCC composed mainly of male cells in a sex-mismatched female kidney transplant patient (Aractingi *et al.* 2005). Unavailablity of information regarding previous blood transfusions or donor-

specific information makes it impossible to exclude a haematopoietic origin of this neoplasm. It is conceivable that the systemic reach of haematopoietic cells enables them access to tissues that they then can engraft in upon stimulation by local biochemical cues.

We performed the first large-scale study examining donor-cell contribution to skin tumours of kidney transplant patients. The absence of microchimerism in CD45-positive cells, as well as the previous observation of the lack of epithelial engraftment of male cells in multiparous control females in our laboratory (Alison *et al.* 2000) led us to the conclusion that the Y-positive cells in this study are indeed donor-derived and not from previous blood transfusions or male pregnancies. The cell type of origin of the donor-derived cells could not be determined and might well be haematopoietic cells carried over with the grafted kidneys.

Consistent with the findings by Aractingi *et al.* (2005), approximately 50% of the patients in our study showed the presence of Y-positive cells. However in contrast to their study, we did not observe tumours that were entirely derived from the donor and found engrafted cells only sporadically distributed, comprising 0.5-6.5% of all epithelial cells in skin tumours and the normal epidermis. Similar levels of engraftment, between 2-8%, have been previously reported in the normal skin with no indication of GvHD (Korbling *et al.* 2002, Hemmati *et al.* 2002). However, other groups could not find any epithelial donor cell-engraftment in the human epidermis (Cogle *et al.* 2007, Murata *et al.* 2007). Like other researchers that studied epithelial engraftment, we could not detect any events of cell fusion.

A comparison of the levels of donor-cell engraftment between our HPV mouse model and the human transplant patients revealed that engraftment of transplanted cells into the tumour and normal epithelia did not differ between the two studies. The stroma was the preferred tissue compartment for incorporation of donor-derived cells. This could be due to stronger stromal cues for the recruitment of donor-derived cells or the higher accessibility of the tumour stroma to circulating leukocytes. Stromal

engraftment also appeared to be significantly higher in human patients than in mice. Although the history of GvHD in our patient pool is not known, immunosuppression, more pronounced inflammatory responses and consequently a larger amount of infiltrating donor-derived leukocytes may contribute to this result.

The extent of donor cell-engraftment appeared to correlate with tumour status, showing significantly less engraftment in premalignant lesions than in lesions that had progressed to the malignant stages. This observation can be explained if progressive stages of tumour development are viewed as higher degrees of injury, where engraftment of donor-cells is known to be enhanced.

Donor-derived cells engrafted as part of the epithelial tumour component and as stromal myofibroblasts; they were susceptible to HPV-infection and contributed to CD34-expressing cells. CD34 has been known for many years as a stem cell marker for haematopoietic stem cells (Berenson *et al.* 1988). It is also expressed on endothelial cells (Fina *et al.* 1990), marks dermal stem cells residing in the bulge of the hair follicle in canine and murine skin (Tumbar *et al.* 2004, Pascucci *et al.* 2006) and recently, it has been shown that its expression in hair follicles is required for skin tumour development in mice (Trempus *et al.* 2007). Finding Y-positive CD34-expressing cells in the tumour stroma suggests that donor-derived cells were able to contribute to tumour-associated blood vessels. The absence of single CD34-expressing cells without any apparent association to blood vessel-like structures may be an indication that donor-derived cells are not likely to engraft in the skin as stem cells.

8.3.2 The impact of the microenvironment on transplanted cells

One of the goals in this thesis was to shed more light onto the reciprocal impact that transplanted cells and host cells exert on each other. This is not only an important issue in transplant medicine, associated with harbouring potential risk factors and additional side effects for patients, but can also help in the development of new treatment strategies. It is well documented that the microenvironment provided by the stem cell niche influences the function of stem cells to a great extent (section 1.1.1). On the other hand, it has also been shown that engrafted BM-derived cells can contribute to epithelial and myocardial healing and thus exhibit a healing influence on their surroundings (de Macedo Braga *et al.* 2007) or conversely form entire donor-derived tumours (Barozzi *et al.* 2003). It is not clear what role the genetic predisposition of host and donor cells plays in those reciprocal effects. For example, is it possible that a genetic predisposition for cancer development of the donor cells follows its phenotypic destiny and causes cancer development in the host and thus presents an inherent risk of transplantations and *vice versa*, is it possible to reverse an adverse genetic predisposition by transplantation of genetically-normal BM cells?

We used a mouse model of skin carcinogenesis and C57/Bl 6 wt mice for reciprocal BM transplantations after lethal irradiation, and additionally applied epithelial wounding in some of the experimental groups to test the effect of injury. With respect to the extent of BM engraftment, our results confirmed previous findings from our laboratory and others: BM cells engrafted into the normal epidermis, skin tumours and their surrounding stroma in a functional and proliferating manner in the absence of cell fusion. Expression of cell type-specific surface markers together with the ability to proliferate (expression of Ki-67) confirmed that the cells fully transdifferentiated and are capable of division, suggesting that the engraftment did not merely represent a form of cell mimicry. Consistent with previously-published reports from a mouse model of pancreatic insulinoma, we observed a higher efficiency of donor cell-engraftment in the tumour stroma than in the bulk of the tumour or the normal epithelium (Direkze *et*

al. 2004). Interestingly, we could not detect a higher engraftment in skin tumours than in the normal mouse epidermis, contrasting with our findings in the skin tumours of kidney transplant recipients, where the degree of malignancy correlated with the percentage of engrafted donor-derived cells. However, the HPV8 mouse model is distinguished merely by benign papilloma formation, but tumour progression and malignancy is rarely observed, preventing a direct comparison to the scenario in human carcinogenesis.

Our results did not reveal an influence of the genotype of engrafted BM-cells and highlighted the importance of the local microenvironment on individual cells. Incorporation of wild type cells did not reverse or decrease the expected host phenotype. The cells behaved as a proliferative (Ki-67 expression) part of the tumour and moreover, the latency of tumour formation was even decreased compared to the control group recipients of HPV8-BM. This might be due to adverse physiological reactions and recognition of the genetically different BM. On the other hand, we could not detect formation of larger clones or HPV8+-derived tumours in wt mice, This may indicate that donor cells in both, the human study and our mouse model did not engraft as stem cells. Future research involving mouse models of chronic injury and higher degrees of malignancy may reveal different patterns of engraftment.

In summary, our results indicated that genetic predisposition of transplanted cells may not present an additional risk factor to transplantation medicine and that the effect of the local microenviroment on individual cell function is larger than *vice versa*.

8.3.3 Mechanisms of engraftment and development of donor-derived tumours

It has been previously shown that the development of donor-derived neoplasms might be dependent on the engraftment of donor cells in the stem cell niche as functioning new tissue resident stem cells, for example due to ablation of the original tissue stem cells upon chronic inflammation (Houghton *et al.* 2004). This theory is supported by the widespread belief that tumours are hierarchically-organised and have their origin in a long-lived cell with sufficient lifespan to acquire carcinogenic mutations, such as stem cells. However, when no chronic inflammation is present, donor cells do not appear to be able to engraft as stem cells in most tissues, and it is not known whether humans may have additional barrier functions preventing the engraftment of 'foreign' stem cells in other tissues. It is conceivable however, that the haematopoietic stem cell niche is less localised and may permit donor-derived cells to incorporate as stem cells and finally act as leukaemia-initating cells. Based on our results, we suggest that donor-derived cells capable of plasticity are distributed with the blood stream to the skin, where they are stimulated by the cues from the local environment to transdifferentiate into various cell types. The newly-engrafted cells contribute to the tissue as differentiated, non-proliferating cells, imitating the differentiated cells of their surroundings in phenotype and function. Thus, while they may contribute to post-transplant skin tumours, they are unlikely to play a role in generating or driving neoplastic formation or progression even when genetically predisposed for tumour development. HPV-infection does not appear to confer sufficient damage to the skin to cause ablation of skin stem cells und subsequent substitution by donor-derived cells. We cannot determine whether the donor-derived cells are haematopoietic cells within the transplanted organ or a separate cell population capable of displaying plasticity.

8.4 LINEAGE RELATIONSHIPS IN THE SKIN AND DEVELOPMENTAL PATHWAYS IN SKIN TUMOURS

Lineage relationships in the skin and the development of skin cancers are much disputed topics. By employing a histochemical assay for activity of the mitochondrially encoded cytochrome c oxidase, successfully demonstrated in our laboratory as a reliable marker of clonality in intestinal crypts, and additional sequencing of the mt genome, we contributed information to several concepts of skin tumour biology. In accordance with the literature, BCCs were generally found to be monoclonal, whereas SCCs were polyclonal. However, in some BCCs, the enzyme assay showed clonal heterogeneity, which could indicate genuine polyclonality within the same tumour or the perceived polyclonality could reflect the simultaneous presence of more than one distinct tumour. Alternatively, the polyclonal pattern could represent subclonality following the acquisition of genomic mutations at later stages of tumour development (Asplund *et al.* 2005) or be based on heteroplasmy within the founding CSC. The latter three are more conceivable scenarios, not only because of bigger patch sizes than the ones observed in SCCs, but also due the high sequence homology between the cytochrome-deficient and competent patches suggesting that the distinguishing mutation must have occurred at a later developmental stage. Additionally, BCCs generally seem to be of monoclonal origin both, in our studies, as well as in the literature.

Controversy exists regarding the stromal origin of BCCs. Xenograft studies suggested that BCC generate their own 'specialised stroma', interspersed with cells derived from the host (Hales *et al.* 1989, Stamp *et al.* 1988), whereas other researchers believe that BCCs are embedded in a connective tissue stroma at least in part of polyclonal origin (Asplund *et al.* 2005). Our results display cytochrome oxidase-deficiency in BCCs, which extended to their surrounding stroma. Although the common origin has not been molecularly confirmed, the consistency of the results obtained in all other tumour areas indicate that BCCs and their stroma are derived from a common ancestor. This supports claims that BCCs generate their own

'specialised' tumour stroma, rather than recruiting a polyclonal stroma from the host as seems to be the case with most cancers.

Researchers, based on histological observations, have argued before that BCCs originate from the same SC pool that gives rise to the ORS located in the hair follicle bulge. In this study, we were able to support this hypothesis by showing a BCC showing continuous cytochrome c oxidase-deficiency, extending throughout a histologically normal ORS of a hair follicle to the entire tumour. The common origin of this monoclonal phenomenon was confirmed by mutation analysis of various cytochrome oxidase-deficient areas.

The observations made during this project not only show that the cell lineages of the IRS are developmentally distinct from the ORS, but also that the companion layer is genetically linked to the IRS and can be viewed as its outermost layer. This is important evidence in the debate regarding the genetic origin of the companion layer and supports previous claims ascribing the companion layer to the IRS based on analysis of keratin expression patterns (Gu *et al.* 2007). Taken together, these results suggest that BCCs and SCCs follow distinct developmental pathways originating in separate SC pools, which is also reflected by tumour-specific differences in cell lineage differentiation.

8.5 FUTURE DIRECTIONS

The characterisation of cancer stem cells and the developmental dynamics of cancer-originating cells are important and exciting topics not only in the search for new therapeutic targets against cancer, but also in the areas of regenerative and transplantation medicine. Although it is widely accepted that a cellular hierarchy exists in tumours, the markers distinguishing cells within this hierarchical system and separating them from normal stem cells are still not clearly defined. The vast amount of contrasting evidence makes one wonder whether the concept of a 'cancer stem cell signature' is a realistic idea or whether the variety of cancer mutations and types of cancer render every cancer with an individualistic set of characteristics that

does not allow for a universal cell fingerprint to be found. Our observations that SP cells are not enriched in stem cells, despite being negative findings, are a step forward in the attempt to understand cancer stem cells. At the start of our experiments, the SP phenotype appeared as the most promising marker for the identification of stem cells and was regarded as a potential universal stem cell marker. Our results, together with other recently published reports have shown that this is not the case. However, an interesting feature in our SP fraction was the distinct CD34-negativity. The behavioural impact of this phenotype has not been elucidated and would necessitate further research.

Our results indicated that CD44 and CD133, as well as their combination may not be useful cancer stem cell markers in colorectal carcinomas. However, other researchers have obtained contrary results. It is conceivable that the results reported in those studies apply to individual cancers and not universally to colon cancers, but repeating our experiments in a different mouse model such as NOD/SCID mice and changing the route of cell administration to renal capsule injection might be useful to validate our findings and exclude the technique as a potential confounding factor for our results.

In this thesis, the first large-scale study is presented, examining donor-cell engraftment in posttransplant skin tumours of kidney transplant recipients. We conclude that donor cells engraft as fully differentiated cells, through biochemical cues triggered to transdifferentiate and imitate the local surrounding cells in phenotype and function. Our results suggest that while donor-derived cells can contribute to various tumour components, they generally do not play a role in tumour development or progression. We showed that HPV-infection does nor confer enough tissue damage to cause ablation of resident stem cells and donor-derived stem cell recruitment. While our results indicate that organ transplantation does not carry an inherent risk for tumour development in the recipient in normal transplant tissue conditions, further studies should be undertaken to examine engraftment of donor cells in circumstances of chronic tissue damage and

the potential of donor-derived tumours in such circumstances. Additionally, while the HPV8 mouse model used in my experiments provides insight into the impact that genetic predisposition of cellular environment and transplanted cells have on cancer development. However, the experiments did not aim to replicate the environmental influences and situations that human transplant patients undergo and thus, to model post-transplant development, a different experimental set-up including immunosuppression, UV-irradiation and HPV infection would be the next step.

In this thesis, I provide first experimental evidence that the tumour stroma of BCCs is continuous with, and thus generated by, tumour cells. These observations were based on immunohistochemistry and histochemical enzyme assay results, but should in future be confirmed by molecular analysis on a wider variety of samples.

We show on a histochemical and molecular level that different SCCs and BCCs are evolutionary distinct and develop from different SC pools and reveal several important aspects of lineage relationship in the skin and the development of skin tumours. The experimental evidence of a BCC being genetically continuous with histologically normal ORS is novel and exciting and provides important information to the understanding of BCC development – the most common skin tumour in humans. This finding should in future be validated on a larger number of samples. Understanding the stem cell pool a tumour originates from is one step on the path to a new targeted therapeutic approach.

In summary, I believe that the results presented in this thesis contribute to several aspects of understanding cancer stem cells and the dynamics of cancer originating cells. The dynamics and interactions between transplanted and host cells not only are an important aspect in transplant medicine and understanding the impact of cellular chimerism, but they can be an important tool in the research into developmental dynamics and factors facilitating and influencing cancer development.

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