Stem cells and cancer in the aerodigestive tract

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Abstract

Recently, there have been significant advances in our knowledge of stem cells found in epithelial tissues. In particular, novel stem cell markers have been identified that for the first time identify multipotential cells; a required characteristic of a stem cell. The scarcity of cancer stem cells has been questioned. Current dogma states that they are rare, but novel research has suggested that this may not be the case. Here I review the latest literature on stem cells, particularly so-called cancer stem cells present in tumours of the respiratory tract and colorectum. I discuss current thinking on how stem cells develop into cancer stem cells, how they protect themselves from doing so, and whether cancer stem cells express unique markers that can be used to detect them. Finally, I attempt to put into perspective these latest advances in cancer stem cell biology from the viewpoint of perhaps more effective cancer therapies.

Introduction

Cancer is most likely a disease that begins in normal stem cells; not only do they already posses a self-renewal capability but, significantly in continually renewing tissues, they are located in a stable niche divorced from a cell escalator, allowing them to acquire a critical level of genetic mutation to establish a 'foothold' against the prevailing efflux of transit amplifying cells (TACs) and terminally differentiated cells. In established tumours the evidence that stem cells are at the root of a hierarchical cellular organisation is becoming increasingly persuasive: crystallising in the 'cancer stem cell hypothesis' that posits that most, if not all, tumours are maintained by an antigenically distinct sub-population(s) of perpetually self-renewing cancer stem cells (CSCs) that also give rise to TACs and terminally differentiated cells.

In this review I discuss stem cell biology and cancer in the respiratory and gastrointestinal tracts, both lined, in the main, by simple epithelia that are directly exposed to many of the known carcinogenic agents. Not surprisingly, cancers of these two systems are amongst the most common. I discuss the location and identity of the normal stem cells, the evidence for these cells being the founder cells of neoplastic lesions, critically evaluate the credentials of cells proposed to be CSCs in these systems, and finally outline the implications for therapy if CSCs are the critical targets if tumours are to be eradicated.

Normal adult stems

When cells are continually being produced to replace worn-out or exfoliated cells, then there is often a unidirectional flow of cells, with stem cells at the beginning of the flux [1]. For example, in the colon, stem cells are at the very base of the crypts; this makes sense if the stem cell population is not to be lost in the migratory flow toward the luminal surface. It was widely held that stem cells are slowly cycling but highly clonogenic. Thus, in many tissues, stem cells divide less frequently than TACs. In the mouse, small intestinal stem cells may be located 4–5 cell positions up from the base, and cycle less often than the more luminally located TACs [2]. On the other hand, another population of stem cells has been identified, located at the crypt base (so-called crypt base columnar cells [CBCs]), that are invariably in the cell cycle [3], so maybe this is not a universal property. As a consequence of the perceived slow cycling nature of many stem cells, a common strategy for supposedly identifying them has relied on finding cells that have retained a label (either tritiated thymidine or bromodeoxyuridine [BrdU]), to which all cells had been exposed to briefly. Hence, stem cells would be *label retaining cells* (LRCs), since the more rapidly cycling TACs would dilute the label more rapidly as a consequence of faster cycling – clearly this technique is inappropriate if some stem cells are rapidly cycling.

Stem cells are defined by their ability to produce more stem cells and cells that differentiate. By undergoing asymmetric cell division, both tasks can be accomplished in a single step [1]. Equally well, stem cell numbers would remain constant if only symmetrical divisions occurred provided that each time a stem cell gave rise to two daughter TACs, another stem cell gave rise to two daughter stem cells. A further possibility is a mixture of symmetric and asymmetric cell divisions. Lack of appropriate signalling can lead to stem cell exhaustion as seen in mice lacking Tcf-4, and so unable to form β-catenin/Tcf-4 complexes essential for Wnt signalling in the small intestine [4].

Many putative stem cells have acquired the ability to withstand cytotoxic insults through either efficient enzyme-based detoxification systems or by the ability to rapidly export potentially harmful xenobiotics. If cells are subjected to Hoechst 33342 dye staining and fluorescence activated cell sorting (FACS) analysis, then those that actively efflux the Hoechst dye appear as a distinct population of cells on the side of the profile, hence the name the 'side population' (SP). The SP phenotype is largely determined by over-expression of one of the ABC superfamily of membrane transporters, often ABCG2, and indeed SP cells isolated from mouse oesophagus are enriched in LRCs [5].

ATP Binding Cassette (ABC) transporters are not the only cytoprotective molecules present in adult stem cells; the aldehyde dehydrogenase (ALDH) gene superfamily encode detoxifying enzymes for many pharmaceuticals and environmental pollutants [6]. Using ALDEFLUOR staining of live cells, combined with low side scatter (an indication of undifferentiated cells with few organelles and protrusions), clonogenic, multipotential stem/progenitor cells have been isolated from many tissues. As expected, high expression of ALDH can be detrimental to tumour eradication; cyclophosphamide treatment of human colonic xenografts enriches for $CD44^+ALDH^+$ cells, and these double positive cells are more tumourigenic than cells selected by solely CD44-positivity [7].

A further mechanism that stem cells appear to employ to reduce susceptibility to potential toxins is through low expression of certain cytochrome P450 enzymes, a superfamily of haemoproteins involved in oxidative (phase I) metabolism; in the pulmonary airways there are so-called pollutant-resistant stem cells that are resistant to toxic insult by virtue of low cytochrome P450 enzymes (see below).

There is no doubt that the Wnt signalling pathway is involved in stem cell renewal in many stem cells, but can we exploit this fact to identify stem cells? A consequence of active Wnt signalling is the nuclear localisation of β-catenin, and indeed this has been demonstrated for murine colonic basal cells [8]; however, nuclear β-catenin is also seen in small intestinal Paneth cells, Wnt signals being required for their maturation [9]. On the other hand, Wnt target genes have been exploited for stem cell identification – Lgr5-positive cells in the mouse small intestine giving rise to long-lived clones containing all cell lineages [3]. Bmi1 is a member of the Polycomb Group family of transcriptional repressors, required for the self-renewal of many stem cells, e.g. the premature senescence of murine neural stem cells is prevented by Bmi1, suppressing transcription at the *Ink4a/Arf* locus that encodes $p16^{Ink4a}$ and $p19^{Arf}$ [10]. In the mouse small intestine, Bmi1-expressing cells have been localised to cell position 4–5, counting from the bottom of the crypt, and lineage tracing from Bmi1-expressing cells suggests that these cells can, like $Lgr5^+$ cells, generate long-lived clones containing all the intestinal cell lineages [11]. Musashi-1 (Msi-1) is an mRNA-binding protein that blocks the translation of *m-Numb* mRNA, so having an enhancing effect on Notch signalling. Msi-1 has been advocated as a stem cell marker in the gut; in the rat stomach Msi-1 expressing cells are located just beneath the foveolar region (the putative niche), though there was some expression in parietal cells [12]. Msi-1 also marks putative stem cells in the mouse intestine [13]; interestingly, in the small intestine, not only were the cells at cell positions 4–5 immuno-labelled, but also the CBCs, now known to express Lgr5 and capable of multilineage differentiation [3]. Though Msi-1 has not been considered as a marker of colorectal cancer stem cells, a quite remarkable suppression of tumour growth occurred when a siRNA knockdown of Msi-1 was achieved in a xenografted colorectal cancer cell line [14].

Prominin-1 (CD133) was the first identified member of the rapidly growing prominin family of pentaspan membrane proteins [15]. The specific functions and ligands of the prominins are still relatively unclear, but they are distinct in their restricted expression within plasma membrane protrusions, such as epithelial microvilli [16]. Two antibodies, CD133/1 (aka AC133) and CD133/2 (aka AC141), recognise different glycosylated epitopes, and most studies use CD133/1. CD133's greatest utility has been in the enrichment of cells with tumour-initiating ability (so-called cancer stem cells) in immune-deficient mice from a variety of human solid tumours [17,18]; however, a recent study has found that most normal and malignant colonic

epithelial cells express CD133, and moreover, CD133-negative cells isolated from colorectal liver metastases were at least as tumourigenic as their CD133-positive counterparts [19].

Adult stem cells are thought to reside in a *stem cell niche*, a specialised microenvironment that contains the signalling molecules required to maintain stem cell identity. In the intestinal crypt, for example, stem cells are located towards the crypt base and surrounding stromal cells, the *pericryptal myofibroblasts*, secrete messenger proteins, including members of the Wnt and BMP families, which contribute to cell fate determination and regulate proliferation in the stem cell compartment [1]. However, the importance of the niche has recently been questioned by the fact that murine Lgr5-positive cells can form crypt-like structures *in vitro* in the absence of a stem cell niche [20].

Stem cells in the respiratory and digestive tracts

Respiratory tract

Progress in identifying respiratory tract stem cells has been impeded by the slow turnover of airway and alveolar epithelium, but the consensus view is that there is no single multipotential stem cell for the lung, but rather there are location-specific stem cell zones in the proximal and distal lung (Fig. 1). In the mouse trachea, basal cells scattered in the ducts of submucosal glands expressing high levels of CK5, that were LRCs after a period of BrdU labelling during tracheal injury, are likely stem/progenitor cells for this pseudo-stratified epithelium [21]. Other tracheal basal cells are also present, probably acting as progenitor cells, and expression of p63 appears important for their self-maintenance [22]. The mouse bronchial tree is lined by a number of cell types including basal cells, ciliated cells and non-ciliated cells (serous, goblet and Clara cell secretory protein [CCSP]-expressing {CE} cells). Here, both CE cells and basal cells appear to be able to act as transit amplifying/progenitor cells, and when CE cells are selectively ablated, a major subset of basal cells (basal cells identified by the binding of the lectin *Griffonia simplicifolia isolectin* B_4 [GSI-B_4]) upregulate CK14 and are able to regenerate the entire epithelium [23]. In the bronchiolar epithelium, the stem cells are rare pollutant-resistant CE cells, co-localised with pulmonary neuroendocrine cells (PNECs), normally located in cell clusters termed *neuroepithelial bodies* (NEBs); PNECs can only act as progenitors for more PNECs [24]. Pollutant resistance of CE cells appears to be related to a deficiency in the phase I drug metabolising enzyme CYP450 2F2. At the bronchioalveolar duct junction (BADJ), Giangreco and colleagues [25] have identified other pollutant resistant CE cells, not associated with NEBs, that probably serve a stem cell function in the terminal bronchioles; CE cells that were also LRCs were located within three cell diameters of the BADJ. At the BADJ are rare CE cells that also express the type II pneumocyte marker, surfactant protein C (SP-C), suggestive of both bronchiolar and alveolar differentiation potential, deserving of the appellation bronchioalveolar stem cells (BASCs) [26]. In the mouse, these BASCs also express CD34 and Sca-1, but not the haematopoietic or endothelial markers CD45 and CD31. In the alveoli, type II pneumocytes are widely believed to be progenitor cells, undergoing hyperplasia in response to the loss of the squamous type I pneumocytes, giving rise to type I cells as well as self-renewing. Type II cells are characteristically recognised by the presence of intracellular lamellar bodies and the production of surfactant proteins.

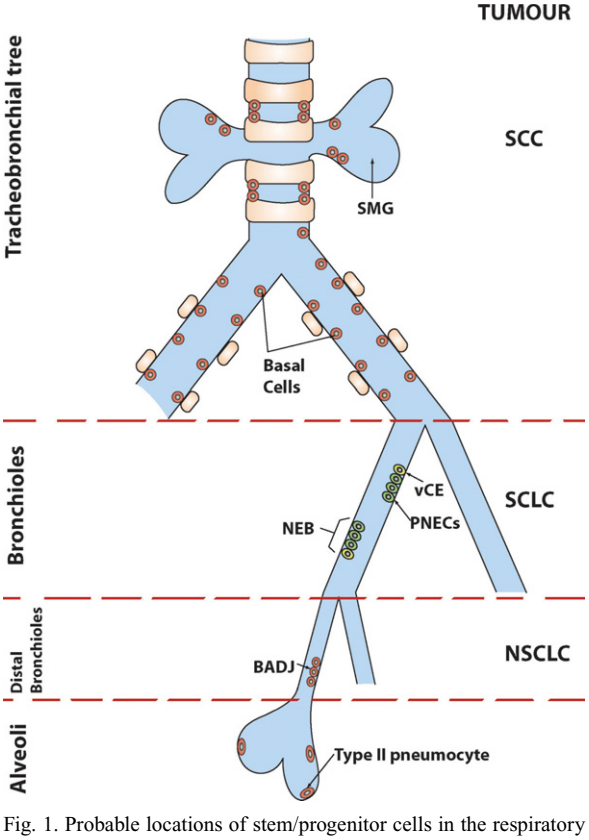

Fig. 1. Probable locations of stem/progenitor cells in the respiratory tree and the tumours that might arise from them. BADJ, bronchioalveolar duct junction; NEB, neuroepithelial body; NSCLC, non-small cell lung cancer; PNECs, pulmonary neuroepithelial cells; SCC, squamous cell carcinoma; SCLC, small cell lung cancer; SMG, submucosal gland; vCE, variant (CCSP) expressing cell.

Digestive tract

The digestive tract is lined by region-specific epithelial coverings, beginning with stratified squamous epithelia lining the oral cavity and oesophagus, progressing to the gastric glands of the stomach, moving distally to the small intestine where the simple epithelial lining is thrown into invaginations (crypts) and finger-like projections (villi), and finally to the colorectum where crypts are still present but villi are absent.

In the mouse, we have already noted LRCs in the basal layer of the oesophagus [5]. In the stomach, the epithelial lining is folded to form gastric glands; stem cells are thought to be primarily located just below the foveolus (pit) with bidirectional cell flux, cells descending downwards toward the gland base and upwards toward the surface *via* the gastric pit. There are few markers described for gastric stem cells, however, Bjerknes and Cheng [27] established that multipotential progenitor cells exist in the mouse stomach; using a mutagenicity strategy with ethyl-nitrosourea, they created mutant clones in adult hemizygous ROSA26 mice *in vivo* that did *not* express β-galactosidase, and all cell lineages could be found in a single clone – persuasive evidence for the existence of multipotential cells. In the isthmic region of mouse antral glands, a population of LRCs expressing a protein more characteristic of the small intestine, villin, have been found [28]. Lineage tracing from villin-positive cells using Cre recombinase suggested these cells were multipotential, but generally they were proliferatively very quiescent unless exposed to inflammatory cytokines.

As discussed above, in the small intestine of the mouse, debate continues over the relative merits of CBCs and the cells at positions 4–5; CBCs are actively cycling cells, nevertheless they appear to function as stem cells; using a tamoxifen activatable Cre recombinase knocked into the *Lgr5* locus, it has been possible to find long-lived clones derived from Lgr5-positive cells that contained all small intestinal lineages, likewise in the large intestine [3]. A similar strategy based on Bmi1-positive cells also generated multilineage clones, but the Bmi1-positive cells were located at cell position 4–5 [11]. Thus, in the mouse small intestine at least, we appear to have two distinct stem cell populations, rapidly cycling $Lgr5^+$ CBC stem cells and a more slowly cycling $Bmi1^+$ stem cell population at positions 4–5.

Tumour histogenesis from stem cells?

Respiratory tract

Intuitively, we believe that tumours have their origins in stem cells, and the evidence supporting this notion is persuasive. In the respiratory tract, the phenotypic diversity of tumours appears related to their location within the pulmonary tree, most likely reflecting the regio-specific variations in stem cell nature [26]. As Kim and colleagues observed in murine models, it seems that only certain specific locations can foster tumour development, there being a proximal to distal distribution pattern of tumours; moving distally from the trachea are squamous cell carcinomas (SCCs), small cell lung carcinomas (SCLCs) and bronchiolo-alveolar carcinomas (BACs)/adenocarcinomas (Fig. 1). We cannot be quite so certain of the origins of human lung cancers. The notion that there may be pro-oncogenic stem cell niches is reinforced by the fact that in murine models where there has been global knock-down of a tumour suppressor gene (e.g. *p53*) or up-regulation of a proto-oncogene under the regulation of a widely expressed lung-specific promoter, in essence a 'field cancerization' effect, there has not been a similarly wide distribution of tumours produced. For example, *K-RAS* mutations are very common in human lung cancer, but murine models with widespread *K-ras* mutations only result in atypical adenomatous hyperplasia and eventually adenocarcinoma in the bronchioalveolar region [29]; related it seems to expansion of self-renewing, multi-potent BASCs in the BADJ [26].

Human SCLCs localise to mid-level bronchioles, and express a number of neuroendocrine markers including calcitonin gene-related peptide (CGRP) normally expressed by PNECs in NEBs. As such, the consensus view is that SCLCs have origin in PNECs. Murine models that delete both *Rb* and *p53* demonstrate hyperplasia specifically in the microenvironment of NEBs with resulting metastatic SCLCs [30]. The proposed relationship between PNECs and SCLC is further strengthened by the fact that the Hedgehog and Notch-delta pathways both have roles in PNEC growth and SCLC progression [31,32].

Squamous cell carcinomas (SCCs) generally occur in the proximal airways and appear to develop in a step-wise fashion beginning with basal cell hyperplasia, progressing to squamous metaplasia, dysplasia, carcinoma *in situ* and finally invasive SCC [33] (Fig. 2A). An origin from CK14-positive basal cells found either in the submucosal gland ducts or intercartilagenous boundaries is consistent with their increased numbers in mouse models of SCC, and the

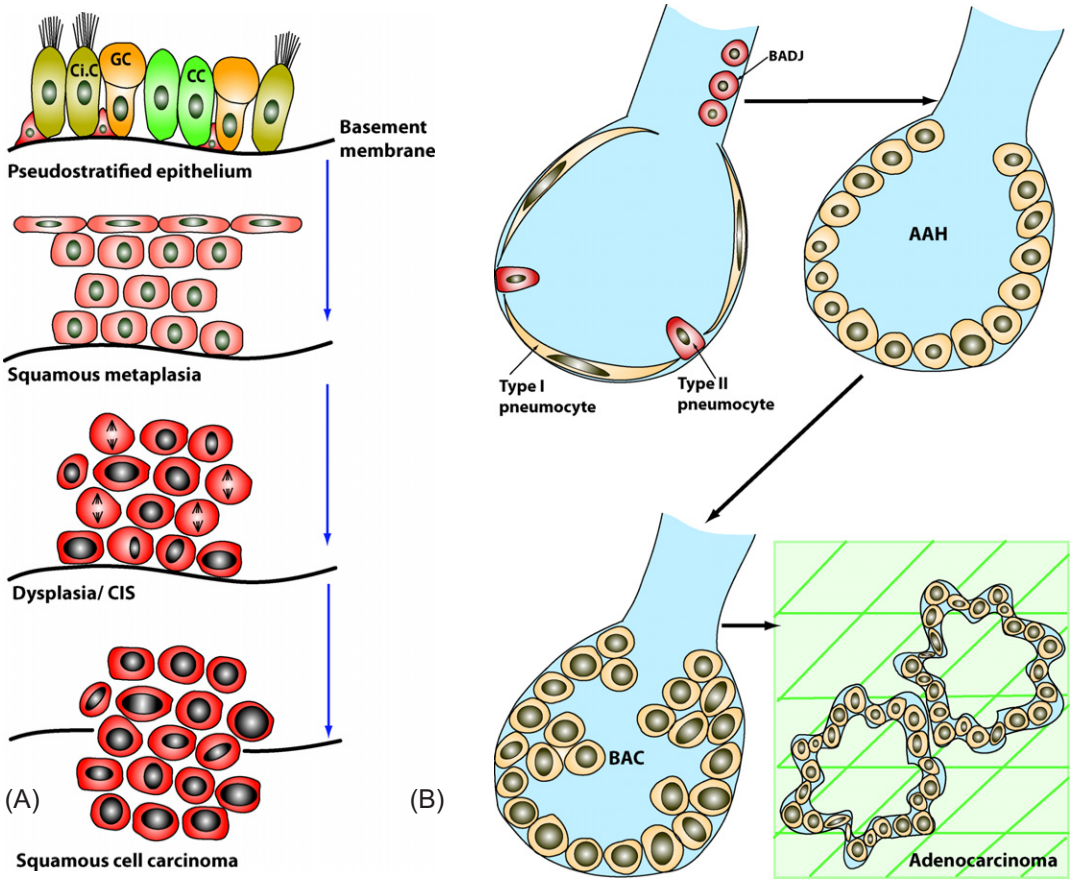

Fig. 2. Simplified cartoons illustrating the major recognisable stages in the histological development of (A) squamous cell carcinoma (SCC) and (B) adenocarcinoma, presumed to reflect the multistage nature of lung cancer development. For detailed morphological descriptions the reader is referred to ref.33. The development of SCC begins in the upper airways with basal cell hyperplasia, proceeding to squamous metaplasia, increasing dysplasia resulting in carcinoma *in situ* (CIS), and finally invasive squamous carcinoma. Peripheral adenocarcinoma may have its origin in BASCs at the BADJ, and proceeds through atypical adenomatous hyperplasia (AAH), bronchioloalveolar carcinoma (BAC) and finally to invasive adenocarcinoma. AAH is thought to be equivalent to an adenoma in the adenoma-carcinoma sequence. CC, clara cell; CiC, ciliated cell; GC, goblet cell.

fact that they occur in the same locations where SCCs arise [34].

Adenocarcinomas sometimes co-express airway and alveolar traits, e.g. CCSP and SP-C, and together with the observed expansion of BASCs prior to tumour formation in mouse models, the evidence strongly suggests that many adenocarcinomas arise from BASCs at the BADJ [26,35], although an origin from Clara cells or type II pneumocytes is also possible. Likewise, many CCSP or SP-C promoter-driven murine models of lung adenocarcinoma produce mixed phenotype tumours located close to terminal bronchioles resembling human bronchoalveolar carcinomas (BACs) [29], again suggestive that the cell of origin of adenocarcinoma and BAC resides at or near the BADJ (Fig. 2B). The Polycomb repressor Bmi1, often over-expressed in NSCLC, is required for normal stem cell maintenance and tumourigenicity in many tumours, functioning, for example, by blocking expression of $p19^{arf}$, a protein that normally sequesters mdm2 and raises p53 levels. In the *K-ras* initiated model of lung adenocarcinoma, loss of Bmi1 inhibits tumourigenesis by blocking prior expansion of BASCs [36].

Digestive tract

The origin of colonic adenomas in man has been contentious, with two main theories – the top-down and bottom-up models (Fig. 3). The top-down model was based upon the frequent observation of dysplastic cells solely at the luminal surface of the crypts [37], along with apparent retrograde migration of adenomatous cells from the surface to the base of the crypt. Only these upper crypt cells showed prominent proliferative activity and nuclear localisa-

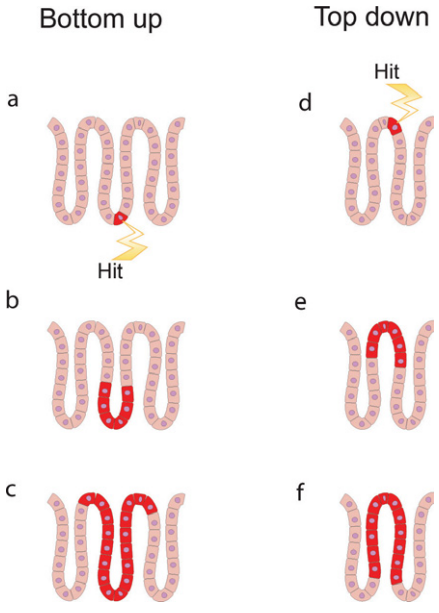

Fig. 3. Top-down or bottom-up growth of colorectal adenomas? Bottom-up – the stem cell, located in the crypt base, undergoes *APC* mutation (A). The mutated cell proliferates (B) and spreads to the top of a crypt to form a monocryptal adenoma (C). Initial further expansion is by crypt fission; based on Preston and colleagues [38]. Top-down – the initial transformation event occurs in a cell in the intercryptal zone (A) and then spreads laterally and downwards (B) eventually filling the whole crypt (C); adapted from Shih and colleagues [37].

tion of β-catenin. These observations are not easily reconciled with the conventional view of the stem cell origin of cancer, and the authors proposed two possible explanations to explain their findings. Firstly, they considered a relocation of the stem cell area to the intercryptal zone, and secondly, they suggested that a mutated stem cell migrates from the base of the crypt to the luminal surface before expanding laterally and downwards. The bottom-up model involves the recognition of the earliest lesion in tumour development, the *monocryptal adenoma*, where the dysplastic cells occupy an entire single crypt. Analysis of tiny (<3 mm) adenomas in familial adenomatous polyposis (FAP) patients showed increased proliferative activity and nuclear β-catenin translocation in morphologically dysplastic cells from the crypt base to the luminal surface [38]. Additionally, there was a sharp cut-off between the dysplastic surface epithelium with nuclear β-catenin expression and the normal mucosa in a neighbouring unaffected crypt. The observation of an increased, asymmetrical crypt fission index in adenomatous tissue led the researchers to propose the bottom-up model – an abnormal stem cell clone with a growth advantage expands from the stem cell niche at the crypt base, to fill an entire crypt. Thereafter, initial spread is by crypt fission to form an *altered crypt focus* (ACF), with top-down spread undoubtedly occurring in slightly larger lesions [38].

In experimental murine models, a direct involvement of stem cells in adenoma formation has been demonstrated, in this case the $Lgr5^{+}$ CBCs [39]; specific deletion of the *Apc* gene in *Lgr5-EGFP-CreER^{T2}/Apc$^{flox/flox}$* mice led to the rapid formation of aggressive adenomas expressing nuclear β-catenin; on the other hand, loss of the *Apc* gene in more differentiated colonic epithelial cells in *Ah-Cre-Apc$^{flox/flox}$/ROSA26R* mice only resulted in occasional small adenomas that grew very slowly. Despite this clear observation, it is worth noting that tumour development in the $Apc^{Min+/-}$ mouse can be greatly suppressed by elimination of Wip1 phosphatase, an enzyme that functions to turn-off the DNA damage response, but the p53-dependent apoptosis occurred at cell positions 4–5, *not* in CBCs [40].

Cancer stem cells

General characteristics

CSCs are believed to have 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 that gives rise to more CSCs. Operationally at present, CSCs are regarded as prospectively purified cells that are more tumourigenic than the bulk or the marker-negative tumour population in a suitable tumour development assay, e.g. after transplantation to NOD/SCID mice. More stringently, a CSC should be a cell that can reconstitute, in a recipient animal, a tumour identical to the original tumour in the patient, which can then be serially xenotransplanted indefinitely. At present, most putative CSCs are identified by their tumour-initiating ability and are thus referred to as tumour-initiating cells (TICs). The conventional wisdom is that CSCs are rare based upon having to xenotransplant large numbers of human tumour cells into immunodeficient mice to further propagate the tumours; however, this might have more to do with a hostile murine microenvironment. For example, as few as 10 mouse lymphoma or AML cells can regularly propagate tumours when transplanted into histocompatible mice, so are all cells in these tumours possible TICs? [41] Using standard immunodeficient mice, the frequency of TICs in human melanoma has been reported to be in the order of 1 in 10^{6} [42], but even single human melanoma cells can form tumours in more highly immunocompromised NOD/SCID/IL2Rγ null mice [43].

The rarity of CSCs has also been questioned in lung cancer; using up to a dozen murine lung cancer cell lines, cell colonies could be regularly generated from randomly selected cells, and when 2×10^5 cells from these clonally-derived colonies were allografted into histocompatible mice, tumours were consistently produced, suggesting that perhaps TICs do not have a unique surface marker signature [44]. Thus, perhaps we need to be more circumspect regarding our thoughts on CSCs, in particular, maybe CSCs are not necessarily rare; nevertheless, they must be effectively targeted for definitive cures since they can often be especially radiotherapy- and chemotherapy-resistant.

CSCs in lung cancer

Evidence for the existence of clonogenic cells in the lung was first described more than 25 years ago in a study by Carney and colleagues [45]. In this study, a small population of cells (<1.5%) isolated from the tumours of both adenocarcinoma of the lung and SCLC patients were able to form colonies in a soft agar cloning assay. As with other solid tumours, CD133 expression features predominantly in the search for CSCs in the lung. In NSCLC, one thousand $CD133^+$ cells could form tumours in SCID mice, but 10^4 CD133-negative cells never did [46]. The CD133-positive cells showed enhanced expression of Oct-4 and ABCG2, and siRNA knockdown of Oct-4 blocked clonogenicity and enhanced chemosensitivity. Expression of Oct-4 has been claimed to occur in BACs, but the data were unconvincing [47]. In both SCLC and NSCLC, a small (<1%) population of CD133-positive cells has been found, with 10^4 of these cells capable of forming tumours in SCID mice with features of the parent tumours [48]. These *in vitro* sphere-forming cells often expressed Oct-4 and Nanog along with CCSP and SP-C. Exploiting the perceived chemotherapy-resistance of CSCs, CSCs have been enriched in a NSCLC cell line by treating with the likes of cisplatin and doxorubicin [49]; 5×10^3 drug selected cells regularly formed tumours in SCID mice. These cells expressed CD133, CD117, and the embryonic stem cell markers SSEA-3, TRA1-81, Oct-4 and had nuclear β-catenin. In the cell line, the SP fraction was 5.2%, but after drug treatment this was increased to 35%. Clearly, drug resistance and lung CSCs are heavily entwined. In the A549 NSCLC cell line a large (24%) SP has been found, with enhanced resistance to doxorubicin and methotrexate related to ABCG2 activity [50]. In a number of SCLC cell lines, a sub-population (1–4%) of urokinase plasminogen activator-positive cells has been found that were more resistant to traditional chemotherapies such as 5-fluorouracil (5-FU), cisplatin and etoposide, seemingly associated with enhanced MDR1 (ABCB1) activity and CD44 expression, both quite common stem cell markers [51].

CSCs in colonic cancer

Proposed markers for CSCs or TICs in colonic carcinomas have included CD44, EpCAM (ESA) and increasingly commonly the membrane-associated protein, CD133. $EpCAM^{high}/CD44^+$ cells have been proposed as TICs for human colorectal adenocarcinomas, with as few as 200 of these cells forming new tumours in the majority (21/28) of immunodeficient mice [52], but many other studies have CD133 as the marker of choice. Ricci-Vitiani and colleagues [53] found that 2.5% of human tumour cells were $CD133^+$ and that 3000 of these cells in Matrigel formed 2 cc tumours within 8 weeks when subcutaneously injected; 100,000 $CD133^-$ cells failed to produce tumours. Likewise, 500–1000 $CD133^+$ tumour cells regularly form colonic tumours when transplanted under the renal capsule of NOD/SCIDs, but 100,000 $CD133^-$ tumour cells do not [54]. On the other hand, isolating human CD133-positive and -negative cells from metastatic deposits, it was the CD133-negative sub-set that produced the more aggressive tumours when xenografted into NOD/SCID mice [19]. Moreover, these authors created a knock-in *LacZ* reporter mouse whose LacZ expression was driven by the CD133 promoter. They showed that CD133 expression was not restricted to the stem cell zone of the crypt, but expressed throughout the entire intestinal epithelium. Moreover, when they crossed $CD133^{LacZ/+}$ mice with $IL\text{-}10^{-/-}$ mice (who develop spontaneous colonic inflammation progressing to adenocarcinoma), the entire tumour expressed CD133! Haraguchi and colleagues [55] reported that human colorectal cancers contain a SP based on expression of ABCG2. However, recent evidence from our group has suggested that SP cells are not enriched for cancer stem cells [56] and SP-enriched or SP-negative cells are both equally able to induce tumours in immunodeficient mice.

The CSC population may in fact be a 'moving target' as cells lose or acquire properties of 'stemness', as seen when Twist or Snail is up-regulated in breast cancer epithelial cells resulting in epithelial-mesenchymal transition (EMT) – remarkably, these mesenchymal-like cells also acquired the widely perceived breast CSC phenotype, $CD44^+CD24^{low}$ [57]. The development of metastasis might well involve

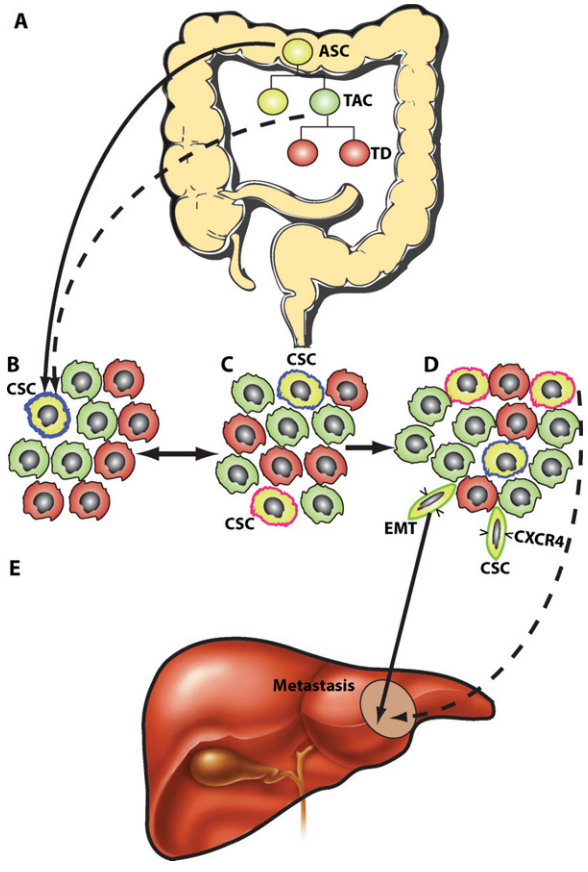

Fig. 4. Current concepts regarding stem cells and tumour evolution in tissues with ordered structure such as epithelia. (A) In normal tissues, adult stem cells (ASC, yellow) self-renew and give rise to transit amplifying cells (TAC, green) that divide several times before undergoing terminal differentiation (TD, red). Many lines of evidence, including direct lineage tracing from genetically marked ASC, indicate that tumours arise from ASCs, though an origin from TACs is also possible. (B) Tumours also have a hierarchical structure, albeit a relatively disorganised one; the cancer stem cells (CSC, yellow, altered chromosomes) may have a single phenotype (blue surface colour denotes a specific surface phenotype, e.g. CD133 in colon cancer) and be rare or relatively common. (C) Genetic or epigenetic events may result in new clones driven by phenotypically diverse populations of CSCs (yellow cells with red surface colour). Further genetic or epigenetic changes may result in some cells undergoing epithelial-mesenchymal transition (EMT, yellow fibroblast-like cell) equipping them with CSC properties, and (D) metastasis may be caused by migrating CSCs detaching from the tumour mass, in particular, these may be the CSCs formed through EMT that may respond to chemotactic gradients by virtue of expression of chemokine receptors such as CXCR4.

the dissemination of CSCs, particularly involving those cells at the tumour margins that have undergone EMT – the so-called *migrating cancer stem cells* [58]. This process may be aided by the expression of chemokine receptors on CSCs as observed in pancreatic cancer [59]. The question of whether tumour heterogeneity is due to distinct clones from different CSCs or whether CSCs, like their normal counterparts, are multipotential, is of fundamental importance. In colorectal cancer (CRC) this question seems to have been answered; in a CRC cell line and from primary CRC, clonal populations have been derived that subsequently recapitulated the heterogeneity of the original tumours when transplanted in nude mice, exhibiting enterocytic, neuroendocrine and goblet cell differentiation – all from a single cell [60,61]; these concepts are depicted in Fig. 4.

Implications for treatment

For tomorrow's oncologist, a variety of druggable targets and strategies related to CSCs present themselves and include:

- Wnt signalling
- Hedgehog signalling
- Notch/Delta signalling
- mTOR
- ABC transporters
- Targeting the stem cell niche
- Small molecule cancer therapeutics (microns, tyrosine kinase inhibitors)
- Gene therapy

Proliferation of CSCs is likely to involve dysregulation of the pathways present in normal stem cell self-renewal such as the Wnt-/β-catenin, PTEN, Notch and Hedgehog pathways, as well as the products of the *Bmi1* and other polycomb genes. In many tissues, key regulators of stem cell renewal appear to be members of the Polycomb group protein family of transcriptional repressors (Bmi1, Rae28, Mel-18). Bmi1 targets genes such as $p16^{INK4A}$ and $p14^{ARF}$ preventing stem cell senescence by respectively maintaining cyclinD/Cdk4 signalling and Mdm2 destruction of p53. Bmi1 is in fact a downstream target of the morphogen Sonic hedgehog through the latter's activation of the Gli family of latent transcription factors. Shh acts on the receptor complex of *Patched* and *Smoothened* (SMO), blocking the restraining influence of *Patched* on SMO, resulting in SMO signalling activating the Gli family of transcription factors and so activating target genes like *Bmi1*. Inhibiting the action of SMO with the antagonist cyclopamine is a highly effective strategy against some cancers. It has been claimed that almost all SCLCs ubiquitously express Bmi1 [62] and antisense *Bmi1* RNA therapy reduces proliferation of A549 lung cells [63]. The Notch family of receptors is also critical for stem cell self-renewal. Engagement of ligands of the Delta and Jagged families causes cleavage, mediated by the γ-secretase protease complex, of

the intracellular portion of Notch and its translocation to the nucleus where it binds to the transcription factor CSL, changing it from a transcriptional repressor to an activator. The use of γ-secretase inhibitors may have utility in cancers where Notch-signalling is inappropriately activated [64].

Apart from the renewal and proliferation pathways, there are many other potential molecular targets relating to CSCs. As many tumours have SP fractions, almost certainly enriched for CSCs, targeting ABC transporter activity will be an obvious strategy for overcoming chemotherapy-resistance as well as directly eradicating stem cells, drug resistance being particularly problematic in SCLCs [65].

Antibody-based targeting of CSCs exploiting the over-expression of the likes of CD133 is another possible approach; CRC $CD133^+$ cells can be resistant to 5-FU and oxaliplatin, probably through autocrine interleukin (IL)-4 signalling; however, blocking the IL-4R sensitises the cells to drug-induced apoptosis [66]. CSCs may reside in areas rich in blood vessels, so-called 'vascular niches'. So while anti-angiogenic therapy is not new, used to debulk tumours through disruption of their blood supply, the destruction of the CSC niche adds a new twist to the story. Small molecule therapeutics that target growth factors, growth factor receptors and their kinases, and more specific tyrosine kinase inhibitors such as imatinib that target c-Kit-positive cells, are also gaining widespread usage. The use of small interfering RNA (siRNA) has been very effective in reducing the growth of xenografted colorectal cancer cell lines, targeting CD44 in HT-29 cells and Msi-1 in HCT116 cells [14,67].

Conclusions

Novel markers of tissue stem cells have recently been proposed that are able to identify cells that are multipotential in nature, including Lgr5 and Bmi1. Although further research is required to demonstrate these molecules as definitive stem cell markers within human tissues, work done in transgenic mice has opened up their potential. Whether or not such the same cell markers can be used to identify tumour-initiating cells in cancer patients is unclear. Cancer stem cells were thought to be rare; however, recent studies have shown that these may be far more common within the tumour than was initially thought, at least in melanoma.

Conflict of interest statement

None declared.

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