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INVITED REVIEW

Cancer stem cells – new and potentially important targets for the therapy of oral squamous cell carcinoma

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There is increasing evidence that the growth and spread of cancers is driven by a small subpopulation of cancer stem cells (CSCs) – the only cells that are capable of long-term self-renewal and generation of the phenotypically diverse tumour cell population. Current failure of cancer therapies may be due to their lesser effect on potentially quiescent CSCs which remain vital and retain their full capacity to repopulate the tumour. Treatment strategies for the elimination of cancer therefore need to consider the consequences of the presence of CSCs. However, the development of new CSC-targeted strategies is currently hindered by the lack of reliable markers for the identification of CSCs and the poor understanding of their behaviour and fate determinants. Recent studies of cell lines derived from oral squamous cell carcinoma (OSCC) indicate the presence of subpopulations of cells with phenotypic and behavioural characteristics corresponding to both normal epithelial stem cells and to cells capable of initiating tumours *in vivo*. The present review discusses the relevance to OSCC of current CSC concepts, the state of various methods for CSC identification, characterization and isolation (clonal functional assay, cell sorting based on surface markers or uptake of Hoechst dye), and possible new approaches to therapy.
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Cellular heterogeneity of oral squamous cell carcinoma (OSCC)

The heterogeneous nature of OSCCs has been demonstrated by histological, phenotypical and karyotypical analyses (Bryne *et al*, 1998; Bankfalvi *et al*, 2002; Tremmel *et al*, 2003). Heterogeneity has been mainly attributed to a process of clonal expansion in which various clones are continuously generated, due to genetic changes, with the daughter cells of more dominant clones overtaking the cells of the other malignant clones in a wave-like fashion (Tabor *et al*, 2001). However, there is an increasing awareness that not all heterogeneity among the cancer cells is the result of genetic heterogeneity and that, within a single tumour clone, cells have significantly different abilities to proliferate and form new tumours. This has led to the hypothesis that most cells in a cancer have a limited ability to divide and only a small subset of phenotypically distinct cells, the cancer stem cells (CSCs), has the capacity to self renew and form new tumours (Hamburger and Salmon, 1977; Reya *et al*, 2001).

A hierarchical concept of heterogeneity of OSCC

Concepts concerning CSCs have often been controversial but the identification of differential patterns of expression of various cell-surface epitopes and fractionation of tumours into subpopulations of cells has now allowed prospective identification of cells that differ markedly with respect to their ability to proliferate and form new tumors (Al-Hajj and Clarke, 2004). Evidence for the presence of CSCs was first presented for haematological malignancies where only a small subset of cancer cells was found to be clonogenic in culture (Griffin and Lowenberg, 1986). More stringent tests, using *in vivo* models for the transplantation of human acute myeloid leukaemia cells into immunodeficient mice, identified a subpopulation of leukaemia-initiating cells which showed immature features and was prospectively identifiable by its $CD34^+CD38^-$ phenotype (Bonnet and Dick, 1997). Recently, CSCs have been similarly identified in solid tumours of the breast, lung and central nervous system. For human breast cancer

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only a small subpopulation of cells with a $CD44^{+}CD24^{-/low}$ phenotype was able to generate new tumours in immunocompromised mice (Al-Hajj *et al*, 2003), and these 'tumour initiating cells' gave rise to all phenotypically diverse cells present in the original tumours. In glioblastoma and medulloblastoma only cells that express the stem-cell marker CD133 have self-renewing and differentiating ability *in vivo* (Singh *et al*, 2004). In murine lung adenocarcinomas a small subset of cells that exhibits self-renewal and is multipotent in clonal assays was identified (Kim *et al*, 2005).

With regard to oral lesions, it has been shown, using 'organotypic' *in vitro* culture models, that cell lines derived from human OSCC contain only small subpopulations of cells that have clonogenic characteristics (Mackenzie, 2004). Although not corresponding fully to the *in vivo* conditions, 'organotypic' cultures provide a more normal environment than standard cultures for both normal and transformed oral keratinocytes (Costea *et al*, 2005). The survival of only a subpopulation of tumour cells in these conditions indicates the existence of a range of growth potentials within the tumour population that might be related to a stem and amplifying cell pattern present in the OSCC of origin (Mackenzie, 2004). More recent studies show that, even years after their isolation and extensive *in vitro* propagation, human OSCC-derived cell lines retain a range of cell types corresponding to the stem and amplifying cells of normal oral epithelium and that a small subpopulation of cells corresponds to tumour-initiating cells and therefore appears to possess the essential defining properties of CSCs (Locke *et al*, 2005).

In normal oral mucosa, as in other self renewing tissues such as bone marrow and skin, compensation for normal physiological cell loss is ultimately dependent on somatic stem cells, a small subset of cells with phenotypically and behaviourally distinct properties. These cells are endowed with (i) seemingly unlimited capacity for self-renewal, and (ii) the ability to generate cells that differentiate to maintain tissue structure and function (Lajtha, 1979; Weissman, 2002; Tudor *et al*, 2004). The production of differentiating cells in OSCCs, although abnormal, indicates that the structure of malignant tissues reflects, to a greater or lesser extent, the structure of the tissue of origin. Given such structural similarities, the question arises as to the extent to which OSCCs also retain the hierarchical proliferative patterns present in their tissues of origin where self-renewal capacity is restricted to a subpopulation of cells. If OSCCs contain CSCs, a hierarchical structure analogous to the tissue of origin would also be expected. Similar to oral epithelium, their tissue of origin, OSCCs are expected to consist of a hierarchy of three cell types: (i) stem cells that typically divide infrequently but retain an extensive self-renewal capacity, and are capable of generating the phenotypically diverse tumour cell population; (ii) amplifying cells that have a limited capability for proliferation but can amplify the maturing population by dividing several times; and (iii) post-mitotic differentiating or differentiated cells (Tudor *et al*, 2004) (Figure 1).

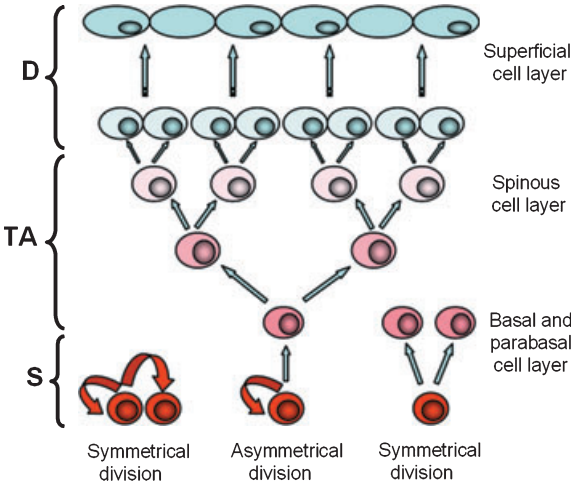

Figure 1 Hierarchical stem cell concept in human oral mucosa. Stem cells (S, red cells) with low proliferative rate but high self-renewal capacity produce, by asymmetric division, a new stem cell (red arrow) and a more differentiated cell (blue arrow) which has a higher proliferative rate but low or absent self-renewal capacity – the transit amplifying cell (TA, pink cells). TA cells divide and differentiate into specialized cells that maintain the tissue (D, blue round cells), and finally desquamate from the epithelial surface (D, blue flattened cells). The asymmetric pattern of stem cell division provides a steady state in which the number of stem cells remains constant while continuously supplying cells into the differentiation pathway. Stem cells can also undergo a symmetrical division to generate two new stem cells or two cells committed to differentiation

Possible origins of CSCs

If the growth of OSCCs depends on a hierarchical proliferative structure, questions arise about the origins of the subpopulation of CSCs. Do they arise through mutations of normal somatic stem cells present within the oral mucosa, or are malignant stem-cell properties acquired as a result of genetic changes and de-differentiation of more mature cells? Given the present uncertainty about the properties of CSCs some other suggestions that have also been made concerning the origin of CSCs cannot yet be excluded.

CSCs may originate from normal somatic stem cells

It has been estimated that three to six genetic events are required to transform a normal human cell into a cancer cell (Kinzler and Vogelstein, 1996; Hahn and Weinberg, 2002). A genetic tumour progression model has been proposed for OSCC (Califano *et al*, 1996), and progressive genetic alterations have also been found to correlate with phenotypic progression to malignancy in OSCC (Califano *et al*, 2000; Gollin, 2001). According to this progression model, the development of most of OSCCs takes months or years. As normal human oral epithelia have a rate of renewal estimated to be about 14–24 days (Squier and Kremer, 2001), most epithelial cells do not exist long enough to accumulate the genetic changes necessary for the development of an OSCC (Braakhuis *et al*, 2004). The hierarchical stem cell structure present in human oral epithelia (Figure 1) indicates that stem cells are the only long-time residents of oral epithelia

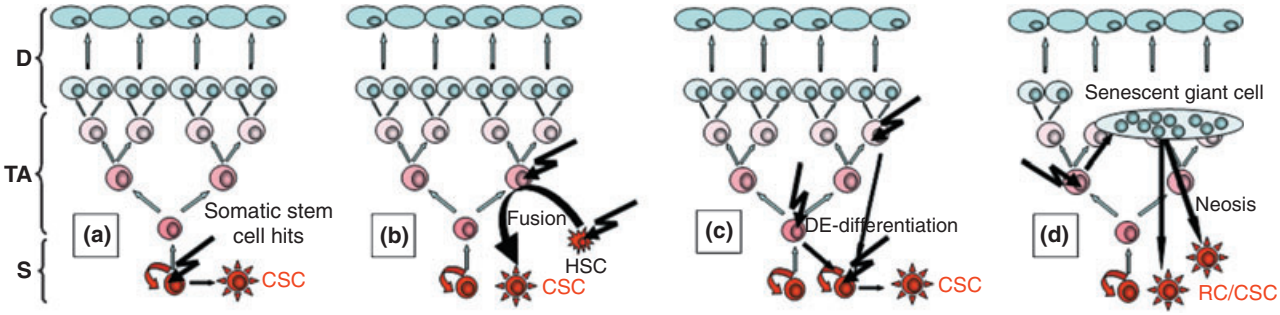

Figure 2 Hypotheses of the origin of cancer stem cells (CSCs) in oral squamous cell carcinoma (OSCC). (**a**) Multiple genetic changes in normal epithelial stem cells. Oncogenic events (black hatched arrow) occur in normal somatic stem cells of oral mucosa, the only cells that reside long enough within the epithelium to acquire the number of genetic changes necessary for transformation and OSCC development to occur. (**b**) Cell fusion between a haematopoietic stem cell and a mutated oral keratinocyte might occasionally occur and lead to genetic instability, aneuploidy and a cell with an altered genome and stem cell properties – a CSC. Alternatively, fusion between a mutated haematopoietic stem cell (HSC) and keratinocytes might give rise to CSCs. (**c**) De-differentiation. Oncogenic events occur initially in an amplifying cell delaying its differentiation and permitting acquisition of additional oncogenic events leading to transformation. (**d**) Neosis. Exposure of cells to genotoxic agents can yield senescent multinucleate giant cells. Their cellular division by cytoplasmic cleavage ('neosis') may result in the formation of multiple small 'Raju' cells (RC/CSC), which seem to display stem cell properties

and, consequently, the only cells able to accumulate the necessary number of genetic changes for malignancy to develop (Owens and Watt, 2003; Perez-Losada and Balmain, 2003; Hunter *et al*, 2005) (Figure 2a).

This model is supported by *in vivo* experimental animal studies of skin carcinogenesis that indicate that exposure to initiating agents produce cellular changes which are retained within the tissue for extended periods of time (Balmain *et al*, 1988; Morris *et al*, 1997). The early observation that only a slowly cycling subpopulation of adult murine epidermal cells retains carcinogens in skin (Morris *et al*, 1986) also sustains the concept that epithelial CSCs are derived from their normal tissue counterparts. Somatic stem cells that have acquired mutations are at higher risk of further progression towards malignancy and the probability of yet further malignant change would be heightened by mutations in genes that affect stem cell division patterns or survival, and thus result in expansion of the population of altered stem cells at risk. In support of this concept, *in vivo* animal experiments show that exposure to ultraviolet light expands clones of epidermal stem cells carrying p53 alterations (Zhang *et al*, 2001, 2005). A similar pattern of clonal expansion of transformed cells has also been demonstrated for human keratinocytes in an *in vitro* 'organotypic' model of human skin carcinogenesis. In this model it was shown that UVB light exposure can induce the intraepithelial expansion of apoptosis-resistant, p53-mutant, and ras-activated keratinocytes at the expense of adjacent normal keratinocytes (Mudgil *et al*, 2003). Displacement of normal stem cells by altered cells is perhaps associated with the development of clonal 'field cancerization' in oral mucosa (Braakhuis *et al*, 2004).

The derivation of CSCs from their normal tissue counterparts is also supported by several other indirect arguments. For example, CSCs and normal somatic stem cells share patterns of molecular expression, for example, of the Notch, Sonic hedgehog and wingless (Wnt) signalling pathways, that are linked to mechanisms for controlling stem cell proliferation and differentiation (Tsai, 2004; Reya and Clevers, 2005). It therefore seems more likely that a newly arising CSC maintains the self-renewal machinery of a pre-existing normal stem cell rather than developing new self-renewal pathways (Reya *et al*, 2001; Pardal *et al*, 2003).

Aneuploidy occurs in some OSCCs and as it is commonly thought to be the result of sequential accumulation of mutations affecting genomic stability, is considered a rather late than an early-stage change during OSCC development (Hemmer and Polackova, 2000). However, normal cells that are exposed to chemical carcinogens may become aneuploid long before they show any specific mutations (Duesberg *et al*, 2004; Duesberg, 2005), and recent studies show that aneuploidy may be present at the early, premalignant stages of OSCC (Maraki *et al*, 2004). A possible alternative explanation for aneuploidy is the fusion of two cells, with aneuploidy resulting from genetic instability that typically occurs after the process of fusion (Hida and Klagsbrun, 2005; Ogle *et al*, 2005). Circulating haematopoietic stem cells (HSCs) have been shown to fuse, both *in vitro* and *in vivo*, with several other cell types, including those of epithelial origin (Wagers and Weissman, 2004). Experimental evidence from animal models shows that carcinoma of the stomach can arise from cells of haematopoietic origin, although these experiments provided no evidence of fusion with epithelial cells (Houghton *et al*, 2004). A recently suggested mechanism underlying the origin of CSCs is fusion between a circulating HSC and a differentiated somatic cell which consequently acquires the self-renewal patterns of the HSC (Bjerkvig *et al*, 2005). Following this concept, the cell fusion process itself might create genetic instability, or alternatively, fusions between mutated somatic cells and HSC might give rise to CSCs (Figure 2b). Direct evidence for such process in the development of OSCCs is lacking, but it does provide a possible mechanism for acquisition of invasive properties of *in situ* carcinomas.

CSCs may originate through de-differentiation of mature cells

Both *in vitro* and *in vivo* experiments suggest that oncogenic events can occur in keratinocytes that are downstream from the most primitive stem cell, and can induce stem-cell renewal capacity and reduce terminal differentiation (Gat *et al*, 1998; Zhu and Watt, 1999). If restricted progenitors can re-acquire the extensive self-renewal potential of stem cells, they could also acquire additional mutations that would lead to transformation (Perez-Losada and Balmain, 2003) (Figure 2c).

In vitro experimental observations that DNA damage can lead to the formation of senescent polyploid giant cells that may be able to escape cell death, divide and give rise to stem-like cells has suggested a novel possible origin for CSCs (Sundaram *et al*, 2004; Rajaraman *et al*, 2005) (Figure 2d). This process, termed 'neosis', is a specific type of cell division of senescent multinucleated cells that produces several small mononuclear cells termed 'Raju cells' which have an extended mitotic lifespan and an ability to generate transformed cell lines. A similar process of genomic instability and micronucleation has been described in cultured senescent breast epithelial cells (Romanov *et al*, 2001), and binucleated and multinucleated cells are seen when normal oral keratinocytes senesce in culture (Kang *et al*, 2000). However, neosis has been described so far only for *in vitro* settings, and whether it is only an artefact of *in vitro* culture conditions remains to be determined. Giant cells have not been identified in OSCCs, although they have been reported for other types of carcinomas where they are described as a secondary reactive event of the host rather than being of neoplastic origin (Donath *et al*, 1997; Alwaheeb and Chetty, 2005; Baydar *et al*, 2006; Willems *et al*, 2005).

Is OSCC stem cell fate controlled intrinsically or by the microenvironment?

Several studies of HCS indicate that their normal self-renewal depends on interactions with neighbouring cells or extracellular substrates that form the so-called stem cell 'niche' (Spradling *et al*, 2001). It has been suggested that tumour development and progression are associated with escape of malignant stem cells from 'niche' dependency (Smalley *et al*, 2005). However, interfollicular epidermis and oral mucosal epithelia seem to have relatively simple stem cell patterns that generate single phenotypic lineages and the nature of stem cell 'niches' has not been clearly defined for such epithelia (Tudor *et al*, 2004). Despite regionally specific patterns of distribution and behaviour of epithelial stem cells *in vivo*, epithelial cells typically show common patterns of *in vitro* clonogenicity that indicate their ability to generate stem and amplifying patterns by intrinsic mechanisms (Tudor *et al*, 2004; Mackenzie, 2005). Similar mechanisms may be associated with the ability of non-malignant oral keratinocytes to suppress the survival, clonal expansion and invasion of OSCC-derived cells (Mackenzie, 2004).

Although localized extra-epithelial environmental influences may not be necessary to determine all aspects of stem cell behaviour in the oral mucosa (Mackenzie, 2005), several studies indicate an important role of the stroma in epithelial stem cell survival and in controlling cell behaviour. For example, the underlying fibroblasts can trigger expansion and invasiveness of early transformed oral keratinocytes that are non-invasive in other conditions (Costea *et al*, 2005). The malignant progression of oral epithelial cells appears to be accompanied by microenvironmental alterations associated with fibroblast activation or conversion to a myofibroblast phenotype (Barth *et al*, 2004; Lewis *et al*, 2004). Fibroblast activation can be induced both by the transforming growth factor-β synthesis of transformed oral keratinocytes (Lewis *et al*, 2004) (Figure 3a), and directly by carcinogen exposure (Rich and Reade, 2001) (Figure 3b). Activated myofibroblasts can provide pro-invasive signals for transformed oral keratinocytes mediated either by diffusible or solid matrix molecules (Matsumoto *et al*, 1989), by direct cell-to-cell contact (Atula *et al*, 1997) or by a combination of both (Costea *et al*, 2006) (Figure 3c). Tumour progression, and the acquisition of a more invasive and metastatic phenotype, is thought to be due to genetic changes induced by new mutational events in transformed keratinocytes (Toruner *et al*, 2004; Roepman *et al*, 2005) (Figure 3d), but additional events cannot be excluded. For example, invasion of myofibroblasts into the epithelial compartment may precede the invasion of malignant epithelial cells into the mesenchymal compartment (De Wever and Mareel, 2003). The co-presence of motile myofibroblasts (Fukumura *et al*, 1998) and fusogenic cancer cells (Duelli and Lazebnik, 2003) may result in cell fusion (Figure 3e), with the new fused cells inheriting both the invasive potential of the myofibroblast and the mutated genome of the transformed keratinocyte. Myofibroblast fusion has been shown in some non-OSCC human oral tumours *in vivo* (El-Labban and Lee, 1983), but whether such mechanisms are associated with the transition from non-invasive *in situ* carcinoma to invasive OSCC remains hypothetical at present.

Identification of CSCs in OSCCs

Normal somatic stem cells and CSCs both form small self-renewing subpopulations but they differ in that self-renewal is highly regulated in normal stem cells but poorly regulated in CSCs. Normal somatic stem cells and CSCs share organogenic capacity but while normal somatic stem cells generate mature normal tissues, CSCs typically generate tissues with aberrant differentiation patterns (Sell and Pierce, 1994). Despite these differences, the parallels existing between normal somatic stem cells and CSCs suggest that the principles of normal stem-cell biology may be usefully applied to studies of CSC identification and their roles in tumour development and progression (Pardal *et al*, 2003). Methods that have been widely employed in stem cell studies include *in vivo* label retention, *in vitro*

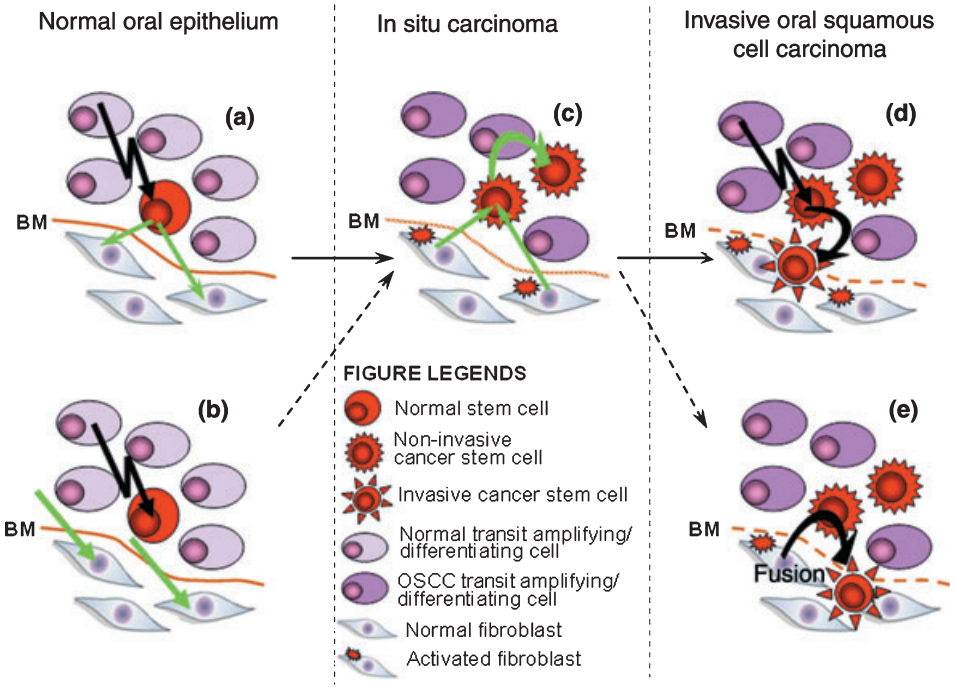

Figure 3 Potential involvement of the stromal microenvironment in oral squamous cell carcinoma (OSCC) progression. Fibroblasts, the predominant cell type in the stroma, change (green arrows) either through stimulation by the genetically transformed keratinocytes (**a**), or directly by exogenous agents such as irradiation or viruses (**b**) into activated fibroblasts (myofibroblasts). These in turn stimulate the transformed keratinocytes (green arrows) possibly influencing stem cell division patterns towards symmetry with increase in the stem cell pool within the OSCC lesion (**c**). Transformed keratinocytes could then acquire further genetic alterations (black hatched arrow) with the evolution of more invasive clones (**d**). Highly motile myofibroblasts may also come into close contact with the highly fusogenic transformed stem cells, and fuse (**e**) to produce a more aggressive cell with the myofibroblast property of high motility and the stem cell property of high self-renewal

clonogenicity and isolation based on fluorescence-activated cell sorting.

Identification of stem cells by label retention

Long-term labelling of cells with nucleotide analogues such as bromodeoxyuridine or tritiated thymidine was initially developed as a method for identifying slow cycling cells, a property expected of stem cells in their normal state. It was anticipated that nucleotide analogues, once incorporated into newly synthesized DNA, would remain within rarely dividing cells and 'label retention' became a commonly used method for stem cell localization (Bickenbach, 1981). However, although label retention can result from proliferative quiescence, more recent findings indicate that it may also be produced by unusual patterns of DNA segregation. Double labelling studies of normal intestinal mucosa indicate that stem cells segregate newly replicated DNA to the daughter cells expected to leave the stem cell compartment (Potten *et al*, 2002). Stem cell retention of the original template DNA may provide a mechanism protecting against their accumulation of genetic errors. Within normal murine epithelia the positions of label-retaining cells (Bickenbach, 1981) correspond to the origins of stem cells lineages detected by transduction with marker genes such as Lac-Z (Mackenzie, 1997), cross-confirming the validity of each method. So far it is unclear how SCSs are distributed in OSCCs and whether CSCs in epithelial tumours are actually slowly cycling, but label-retaining studies of transplanted OSCCs should enable resolution of such questions.

In vitro clonal assays

Haematopoietic stem cells are characterized both by their *in vivo* ability to reconstitute all haematopoietic lineages in lethally irradiated mice and by their unique *in vitro* patterns of growth and differentiation (Huntly and Gilliland, 2005). As *in vivo* assays are particularly difficult to apply to epithelial stem cells (Watt, 2000; Kim *et al*, 2005) more reliance has been placed on *in vitro* clonal assays (Li *et al*, 2004; Mackenzie, 2005). Normal human skin keratinocytes grown *in vitro* have been classified into three subpopulations based on their patterns of clonal expansion (Barrandon and Green, 1985, 1987). Compact colonies of small cells are formed by some individual founder cells and can be repeatedly passaged. Other cells generate irregular colonies containing fewer cells capable of growth on further passage. Yet others form colonies that fail to proliferate further and consist of large flattened cells. These colony forms, referred to as holoclones, meroclones and paraclones, are considered to be derived from stem cells and early and late transit amplifying cells respectively (Barrandon and Green, 1987). Such colony morphologies can also be identified when normal human oral keratinocytes (NOK) are plated at clonal densities (Figure 4a–c) (DE Costea, O Tsimkalovsky, J Wang, OK Vintermyr, AC Johannessen, IC Mackenzie, unpublished data). Cell lines derived from OSCCs also generate marked clonal

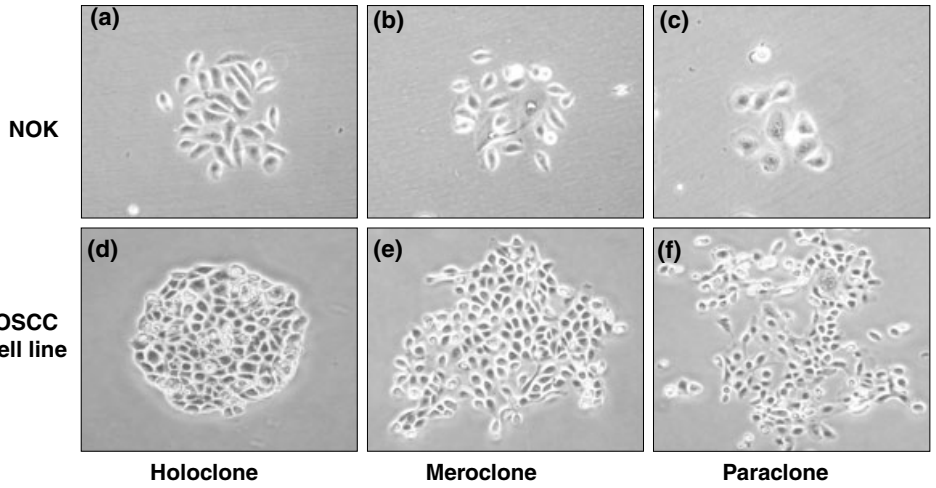

Figure 4 Clonal morphology of primary normal human oral keratinocytes (NOK) and human oral squamous cell carcinoma (OSCC)-derived cell line. (**a**–**c**) Morphology of clones formed by human NOKs isolated and grown on plastic surfaces in keratinocyte-serum free medium (K-SFM). (**d**–**f**) An OSCC-derived cell line grown under similar conditions. Holoclones are characterized by round colony outlines and small, closely packed cells (**a**, **d**). Meroclones have larger and somewhat more flattened cells that remain in contact with each other in the central region but at the periphery of the colony have separated and acquired an ovoid outline (**b**, **e**). Paraclones consist largely of flattened scattered ovoid cells, few of which remain in contact (**c**, **f**). The differences in colony morphologies are readily distinguishable but the continuous gradient of change from one colony form to the next makes precise classification somewhat arbitrary

heterogeneity and form a range of colony morphologies paralleling the holoclone, meroclone and paraclone morphologies produced by normal keratinocytes (Locke *et al*, 2005) (Figure 4d–f). As for normal epithelia, the cells of malignant holoclones differ from paraclone cells in being smaller, more rapidly adhesive and more highly clonogenic. The behaviour and patterns of marker expression of malignant holoclone cells are similar both to normal epithelial stem cells and to tumour-initiating cells and these cells therefore possess the essential defining properties of malignant stem cells (Locke *et al*, 2005). Clonal assays thus seem to provide a robust and reliable method for the identification and isolation of cells with stem cell properties from both normal and neoplastic oral mucosa and can provide systems for the characterization of CSC responses to various factors and therapeutic agents.

Flow cytometry using cell surface markers

Watt and colleagues were the first to attempt prospective identification and isolation of normal epidermal stem cells using cell surface markers and flow cytometric techniques (Jones *et al*, 1993). Studies of cultured human foreskin keratinocytes showed that cells expressing high levels of β1-integrin had higher colony-forming efficiencies, determined over a 2-week period in culture. Extension of these studies to freshly isolated neonatal human keratinocytes further showed that cells expressing high levels of β1-integrin can generate a functional epithelium when grafted onto mice (Jones *et al*, 1995) suggesting that this subpopulation of basal epidermal cells is enriched for keratinocyte stem cells. Sorting cells from neonatal human skin, based on the combined detection of α6-integrin and the transferrin receptor CD71, also isolates a small subpopulation of cells with relative cell-cycle quiescence and high long-term proliferative potential (Li *et al*, 1998; Kaur and Li, 2000) but a limitation of these markers is that they are both upregulated in wound healing and in cell culture (Kaur *et al*, 2004). Other work indicates the importance of an appropriate microenvironment for such assays and that even keratinocytes that have downregulated integrin expression and begun to express cytokeratin 10 are capable of extensive proliferation *in vitro* (Li *et al*, 1998) and can form an epidermis in organotypic cultures (Li *et al*, 2004).

Few reliable stem cell markers have been found for normal oral epithelium (Tudor *et al*, 2004) and most of them, being intracellular markers, are unsuitable for flow sorting (Table 1). However, studies of OSCC-derived cell lines have identified additional markers that may be of use for the isolation of stem cells from neoplastic oral mucosa. In OSCC-derived cell lines, colonies with holoclone morphologies show consistently higher levels of expression of stem cell-related molecules such as β1-integrin, E-cadherin, β-catenin, epithelial specific antigen (ESA) and CD44 (Locke *et al*, 2005). Interestingly, CD44 was the primary marker used to isolate tumour-initiating cells from breast cancers (Al-Hajj *et al*, 2003; Locke *et al*, 2005).

Flow cytometry using Hoechst dye exclusion

Primitive stem cells with long-term proliferating ability in the murine haematopoietic system have the ability to exclude the DNA-binding dye Hoechst 33342 (Goodell *et al*, 1996). This results in a characteristic flow cytometric profile with dye-excluding cells distinct from the main cell population and they are referred to as side population (SP) cells (Figure 5). Mouse mammary epithelial populations that are enriched for progenitor cells have been identified using antibodies against stem cell antigen-1 and have SP properties (Welm *et al*, 2002) suggesting that dye exclusion may be a generalized stem cell

Table 1 Stem cell markers with potential value for sorting CSCs by flow cytometry

Potential stem cell marker	*Suitability*	*Tissue*	*Reference*
β1-integrin (high)	Yes	Normal skin	Jones *et al* (1993), Li and Kaur (2005)
		OSCC-derived cell lines	Locke *et al* (2005)
α6-integrin (high)	Yes	Normal skin	Li *et al* (1998), Kaur and Li (2000)
CD71 (transferrin receptor) (low)	Yes	Normal skin	Li *et al* (1998), Kaur and Li (2000)
CK 15 (high)	No	Normal palatal epithelium	Tudor *et al* (2004)
CK 19 (high)	No	Normal palatal epithelium	Tudor *et al* (2004)
CK 16 (low)	No	Normal palatal epithelium	Tudor *et al* (2004)
CD44 (high)	Yes	Breast cancer	Al-Hajj *et al* (2003)
		OSCC-derived cell lines	Locke *et al* (2005)
ESA (high)	Yes	Breast cancer	Al-Hajj *et al* (2003)
		OSCC-derived cell lines	Locke *et al* (2005)
E-cadherin(high)	Yes	OSCC-derived cell lines	Locke *et al* (2005)
β-catenin (high)	No	OSCC-derived cell lines	Locke *et al* (2005)

CSC, cancer stem cell; ESA, epithelial specific antigen; OSCC, oral squamous cell carcinoma.

property. SP cells have also been isolated from normal mouse and human dermis, but this population appears to have a different cell surface phenotype (low β1-integrin and low α6-integrin) from other putative stem cell populations identified as label-retaining cells (Terunuma *et al*, 2003; Triel *et al*, 2004). However, others have defined stem cell populations on the basis of label retention, clonal expansion, high proliferative potential and epidermal regenerative capacity of cells assayed after sorting by size and Hoechst efflux (Bickenbach, 2005; Larderet *et al*, 2006). SP cells have been identified in a wide range of malignant cell lines (Hirschmann-Jax *et al*, 2004). Work in our laboratory with both OSCC-derived cell lines and primary NOK supports the existence of SP cells as a subset of oral keratinocytes (Figure 5), and functional data to test the validity of the SP population as stem cells is still being gathered (DE Costea, O Tsimkalovsky, J Wang, OK Vintermyr, AC Johannessen, K Mackenzie, unpublished data).

Therapeutic implications of a hierarchical proliferative structure in OSCC

Oral squamous cell carcinoma is associated with severe morbidity and less than 50% long-term survival (Greenlee *et al*, 2000; Shah *et al*, 2003). Despite various new and advanced treatment modalities, the poor prognosis of OSCC has not improved significantly over the last 4 decades (Williams, 2000; Bernier *et al*, 2004; Warnakulasuriya *et al*, 2005). Most of the anti-cancer treatment strategies used today are based on the classical view that the tumour is generated by a population of cells with equal proliferative potentials (Sarraf, 2005). However, a hierarchical pattern of organization of OSCC indicates that successful treatment modalities for the elimination of cancer need to take into account the unusual properties of the CSC subpopulation. Whether CSCs are generally slowly cycling (i.e. are replication quiescent cells) has not yet been determined for OSCCs, but they appear to be slowly cycling in leukaemias (Holyoake *et al*, 2001) and in some epithelial cancers (Xin *et al*, 2005). When cells are out of the cell cycle they are often inherently resistant to chemotherapy and radiotherapy (Maitland and Collins, 2005), and the actions of anti-cancer therapies currently available for OSCC may thus be less effective against potentially quiescent CSCs. With survival of these cells, a full capacity for repopulation of the tumour would be retained (Figure 6a). The effectiveness of the actions of cytotoxic drugs may be also reduced by the high CSC expression of drug transporters which enable rapid exportation of noxious substances from the cytosol (Hirschmann-Jax *et al*, 2004). Characterization of such differential properties is therefore required to monitor the effects of existing therapeutic interventions on CSCs, as well as for the development of therapies that more effectively target the CSC population.

Cellular antigens or signalling pathways that are expressed by the bulk of cancer cells form optimal therapeutic targets only if they are also expressed by the subpopulation of CSCs (Jones *et al*, 2004). The search for novel therapies with specificity for CSCs would be greatly assisted by the identification of differences in gene expression – both between normal and cancer cells and within the heterogeneous cancer cell population (Jones *et al*, 2004). There in fact appears to be differential expression of a wide range of genes between malignant holoclone and paraclone cells derived from OSCCs (Locke *et al*, 2005). This supports the concept that better methods for CSC identification can be developed and that there may be CSC-specific molecules, perhaps associated with the control of stem cell fate pathways, that are suitable for selective therapeutic targeting (Locke *et al*, 2005). Further understanding of the fate determinants of CSCs might also support a concept of 'directed differentiation' for reducing the self-renewal of OSCCs by switching the probability of asymmetric CSC division towards symmetrical division with the production of two differentiating daughter cells and the loss of the pre-existing stem cell properties (Mackenzie, 2005). Reversible mechanisms that lead to the expansion of epithelial stem cell populations appear to occur during normal growth and healing (Watt and Hogan, 2000) and pharmacological manipulation of

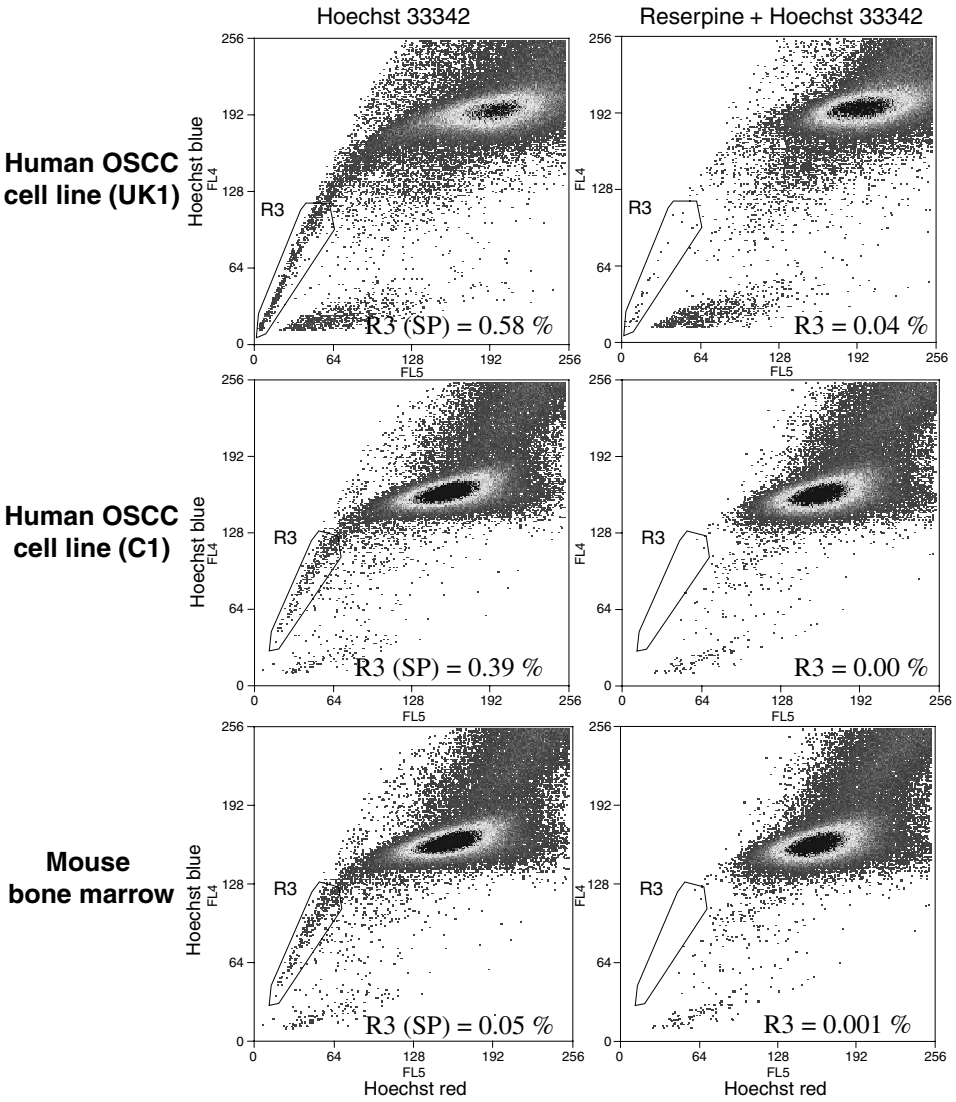

Figure 5 Oral squamous cell carcinoma (OSCC)-derived cell lines have a side population (SP) sensitive to reserpine. OSCC-derived cell lines UK1 and C1 (Locke *et al*, 2005) were incubated at 37°C with 12μg ml^{-1} Hoechst for 120 min. Parallel samples were pretreated with 5 μM reserpine for 15 min prior to Hoechst staining. The protocol has been adapted after the one used for isolation of SP in mouse bone marrow cells which are also shown as a control tissue (Tsinkalovsky *et al*, 2005). The Hoechst fluorescence distribution of human OSCC-derived cell lines revealed a reserpine-sensitive SP

CSC self-renewal appears feasible at least *in vitro* (Sherley, 2002). If such mechanisms could be manipulated to produce a shift in malignant stem cells away from self-renewal, stem cell loss and tumour atrophy would effectively follow (Figure 6b).

As there is some experimental evidence for the role of the OSCC stroma in CSC survival and behaviour, novel treatment strategies might also take into consideration the possibility of modulating the clonogenicity and expansion of CSCs by manipulating the local microenvironment (e.g. the activated carcinoma-associated fibroblasts) (De Wever and Mareel, 2003) (Figure 6b). The principle that co-targeting both tumour cells and their supporting stroma is more efficacious than targeting the tumour alone has been recently shown in a model of human prostate cancer (Hsieh *et al*, 2004). Some authors suggest that targeting the activated cancer stroma may be easier than targeting the tumour itself; tumour stroma-associated antigens have a more restricted expression pattern and have been successfully targeted by passive and active immunotherapy in preclinical models (Hofmeister *et al*, 2006).

Possibly other stem cell properties can also be used to develop novel anti-cancer treatment strategies. For example, normal mesenchymal stem cells (MSC) appear to be attracted by tumour stroma (Brower, 2005). *In vivo* animal experiments indicate that fluorescently labelled human bone marrow MSCs, injected into the carotid artery of mice bearing intracranial xenografts of human glioma, are attracted exclusively to the tumours (Nakamizo *et al*, 2005). Studies of micrometastases of human breast and colon cancers in murine models also suggest that normal MSCs can be used as targeted carriers for cytokines and tumour-killing proteins

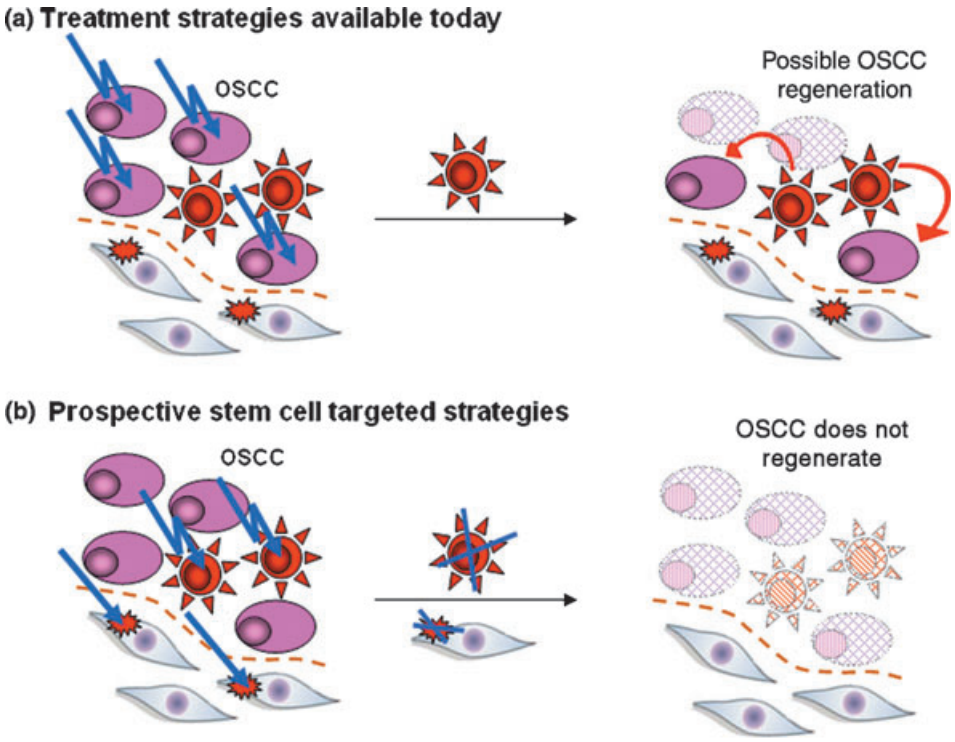

Figure 6 Oral squamous cell carcinoma (OSCC) treatment strategies. OSCCs are heterogeneous lesions that may contain a small population of potentially quiescent cancer stem cells (CSCs) which are not responsive to the treatment strategies primarily directed against proliferative cells, survive treatment, and may be responsible for tumour recurrences (**a**). Targeting and destruction of CSCs will lead to disappearance of the malignant lesion as the tumour mass cannot be replenished in the absence of CSCs (**b**). Targeting reactive tumour stroma may help to eliminate micro-environmental signals that might sustain CSC recovery (**b**)

(Studeny *et al*, 2004; Hung *et al*, 2005), but how species specificity affects the homing of MSCs in these studies is as yet unclear (Wolf *et al*, 2005). *In vitro* experiments indicate that keratinocytes are also able to attract MSCs, but whether this property can be of use in the therapy of OSCCs remains to be determined (Akino *et al*, 2005).

Conclusions

It is becoming apparent that the growth of tumours is associated with stem and amplifying patterns similar to those of normal tissues. Recent evidence indicates that even cell lines generated from OSCCs consistently produce *in vitro* colony patterns unexpectedly similar to those produced by the stem and amplifying cells of normal epithelia. The maintenance of a subpopulation of stem cells during the passage of cell lines indicates that the key stem cell property of asymmetrical division persists, but population expansion indicates that it is shifted towards enhanced stem cell self-renewal (Locke *et al*, 2005). Methods for the destruction of CSCs to eliminate the regenerative capacity of the tumour should provide therapeutic effects that contrast with current unsuccessful therapies. These appear to destroy the main bulk of the proliferating tumour tissue but do not eliminate the regenerative capacity of the therapy-resistant CSCs (Figure 6). A major impediment to the development of such treatment strategies is that methods are still lacking that can readily distinguish CSCs from their differentiating progeny and from their normal tissue counterparts. However, major efforts are being applied to basic stem cell research and it appears likely that new and efficient treatment strategies will emerge to change the clinical outcomes of current OSCC management.

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