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Stem cell patterns in cell lines derived from head and neck squamous cell carcinoma

Lisa J. Harper[1], Kim Piper[2], John Common[1], Farida Fortune[2], Ian C. Mackenzie[1]

[1]*Institute of Cell and Molecular Science and* [2]*Centre for Diagnostic Oral Sciences, Queen Mary's School of Medicine and Dentistry, Whitechapel, London, UK*

The initiation, growth, recurrence and metastasis of solid tumours, including squamous cell carcinoma of the head and neck region, have been related to the behaviour of a small subpopulation of 'tumour-initiating' cells. Cells with stem cell characteristics have also been identified in cell lines derived from cancers and the aim of the present work was to extend examination of such cells. Established cell lines were examined for their patterns of colony morphologies and staining, the presence of a Hoechst dye-excluding 'side population', expression of the putative stem cell markers CD44, CD133 and CD29, and their ability to grow as 'cancer spheroids'. Two cell lines, CaLH2 and CaLH3, recently generated from HNSCC tumour biopsies, were similarly examined. All cell lines showed a holoclone/meroclone/paraclone series of colony morphologies and cell sorting indicated that CD44 marker expression was related to clonogenicity. FACS analysis after exposure to Hoechst dye indicated that the CA1, H357 and UK1 cell lines contain a dye-excluding 'side population', a property associated with stem-like subpopulations. When held in suspension, all cell lines formed spheroids that could be re-passaged. These observations indicate that cell lines derived from HNSCC contain cells with stem cell properties and that such cell lines may provide experimental systems relevant to the behaviour of stem cells present in the tumours of origin and to their responses to therapy.
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Introduction

Head and neck squamous cell carcinoma (HNSCC) is among the 10 most common cancers worldwide (1, 2) and, despite recent acquisition of new basic and clinical information, the overall 5-year survival rate for HNSCC remains as low as 50% (3). More effective approaches to therapy are therefore urgently required. It has now been shown that HNSCC, like many other tumours, contain a subpopulation of 'tumour-initiating cells' that apparently correspond to the malignant stem cells responsible for tumour growth (4, 5). It is likely that local recurrence and metastasis are associated with the early spread of such cells and that current therapeutic strategies may be ineffective in controlling them (6–8). Cancers arise through a process of sequential mutation and clonal selection (9, 10) and the idea that changes that occur in normal stem cells lead to the development of 'cancer stem cells' (CSCs) has been extensively discussed (reviewed in Ref. 11). Six acquired 'hallmarks of cancer' have been suggested (12): limitless cell replication, self-sufficient growth, avoidance of apoptosis, insensitivity to antigrowth signals, sustained angiogenesis, and invasion and metastasis. It has yet to be adequately explored how such changes are related to the stem cell patterns now identified in malignant tissues but it is interesting that the first three of these behavioural hallmarks are likely to depend directly on changes in stem cell behaviour (11, 13).

The continuous regeneration of tissues such as bone marrow and stratified epithelia depends ultimately on a subpopulation of slowly dividing 'somatic stem cells' (14–17). It has been appreciated for many years that cells isolated from tumours differ in their clonogenicities and that such differences might reflect a stem and amplifying cell pattern (18). A rare population of stem cells underlies renewal of the normal haematopoietic system (16) and the 'tumour-initiating' cells responsible for the growth and recurrence of leukaemias represents only a very small fraction of the total malignant cell population (11, 19). Normal epithelial stem cells, like haematopoietic stem cells, typically divide infrequently but have the ability to divide asymmetrically so that each division renews the stem cell itself while generating another cell that undergo a series of amplification divisions to augment the total differentiating population (20). Recently, there has been increasing recognition of

Correspondence: Dr Ian C. Mackenzie, Institute of Cell and Molecular Biology, Queen Mary's School of Medicine and Dentistry, 4 Newark Street, London E1 2AT, UK. Tel: +44 20 7882 7159, Fax: +44 20 7882 7172, E-mail: i.c.mackenzie@qmul.ac.uk
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the relevance of such hierarchical stem cell patterns to the development and growth of solid tumours. During the past 3 years, compelling evidence has been generated for the persistence of stem cell patterns in gliomas and in cancers of the breast, colon and prostate (21–24). A similar pattern has now also been reported for HNSCC (5). Stem cell patterns also appear to persist in cell lines derived from a wide range of malignancies (11, 19, 25–30) including cell lines derived from HNSSC (8, 28).

The early recognition of the potential importance of stem cell patterns of tumour renewal (18) has lead to the realization that successful therapies need at least to include CSCs within their range of fatal actions (6, 18, 31, 32). *In vitro* methods for detecting differential sensitivities of malignant stem cell populations to therapeutic procedures would therefore clearly be of value (18). However, the disordered structure of malignant tissues suggests that normal stem cell patterns may be disrupted and the generation of cell lines from such tissues might be expected to lead to further cellular changes as a result of selection during adaptation to *in vitro* growth. Consequently, the normality of stem cell patterns present in cell lines and the value of information that might be derived from them have been questioned (21). However, given the difficulty of *in vivo* assessment of stem cell behaviour, if stem cell patterns are retained in cell lines such *in vitro* systems are likely to be useful for the investigation of key stem cell properties.

We have previously reported morphological and marker methods for identifying and isolating malignant stem and amplifying cells and demonstrated the persistence of stem cell patterns in cell lines by the heterogeneous patterns of cell morphology, marker expression and clonogenicity corresponding to those of normal epithelia (28). For normal human epithelia, stem cell properties have been assessed by the differing patterns of keratinocyte clonogenicity when isolated and grown at clonal densities (33). Colonies termed holoclones, consisting of small, tightly-packed cells with high proliferative potential are thought to contain stem cells. Meroclones contain larger cells than holoclones and these have less proliferative potential, are not able to self-renew, and appear to correspond to transit amplifying cells. Paraclones, consisting of large, flattened cells, have low proliferative potential and correspond to early differentiating cells. Such clonal patterns are displayed by malignant cell lines with only the cells of malignant holoclones capable of self-renewal (28). Further, normal epithelial stem cells and malignant holoclones share higher expression of molecules such as β1-integrin, β-catenin, e-cadherin and cytokeratin 15 (14, 28, 34). Malignant holoclones also show higher expression of CD44, a marker of the tumour-initiating subpopulations in breast and oral cancers (5, 23). Aims of the present study were to determine whether HNSCC cell lines that have been maintained in culture for many years, and also cell lines newly derived from HNSCC, show additional properties recently reported for the stem cell fractions of other tumours, including of the presence of subpopulations of cells that can exclude Hoechst dye, subpopulations expressing CD133, and the ability to grow in suspension as 'cancer spheres' (24, 26, 30, 35).

Methods

Generation of cell lines

Fresh tumour samples were collected from HNSCC patients with informed consent and samples placed in transport medium (RM + with ×5 antibiotic concentration, see below) for overnight incubation at 4°C. Samples were then washed three times in fresh transport medium with vortexing to remove any lose debris. Samples were cut into pieces approximately 1 mm^2, placed on 6 cm tissue culture dishes and left in a laminar flow hood for 15–20 min to dry and adhere to the dish. RM + medium [consisting of a 3 : 1 ratio of DMEM : F12 with 10% fetal bovine serum (FBS), 1% glutamine, 0.4 μg hydrocortisone, cholera toxin 10^{-10} M, transferrin 5 μg/ml, liothyronine 2×10^{-11} M, insulin 5 μg/ml, epidermal growth factor 10 ng/ml, ×1 antibiotic/antimycotic mixture] was added and the dishes placed in a humid incubator at 37°C and 5% CO_2 with the medium changed once a week. When outgrowth from the tissue pieces occurred, cloning rings were used to isolate the outgrowing epithelial cells, which were removed by trypsinization (0.05% trypsin/EDTA) and plated onto Mitomycin C-treated 3T3 feeder cells, basically according to the methods of Rheinwald and Green (36). As they approached confluence, epithelial cells were passaged 3–4 times on 3T3 feeder cells, then passaged without feeder cells at high density before assessing their ability for growth at clonal densities (260 cell/cm^2). For plating at low densities and as single cells, the RM + growth medium was supplemented with an equal part of the same medium conditioned by growth of subconfluent cells of the same line for 24 h. Each cell population was periodically recloned by plating single cells in conditioned medium under direct vision and expanding fresh populations from a single clone. Once isolated and recloned, newly isolated lines and the pre-existing cell lines CA1, H357 and UK1 (28) were grown in RM + medium to examine their patterns of colony formation. Cells were trypsinized, washed in serum-containing medium, spun down, filtered through a 40 μm filter to provide a single cell suspension, recounted and plated at 260 cells/cm^2 in T25 or T75 flasks and grown for 4–7 days.

Immunocytochemistry

Cells growing in flasks or multiwell plates were fixed in 4% paraformaldehyde, washed with PBS, permeabilized with 0.2% Triton X-100 for 10 min, and blocked with 5% BSA for 1 h. The primary antibodies used were mouse monoclonal antibodies against CD44 and β-catenin (BD Biosciences, Oxford, UK), CD29 (Upstate Biotechnology, Dundee, UK), Epithelial-specific Antigen (Novocastra, Newcastle, UK, Clone VU-1D9), CD133 (ab31448, Abcam, Cambridge, UK) and cytokeratins 6 and 14 (mAbs LL020, LL001, gifts from Prof. Irene Leigh). Cells were incubated with

primary antibodies overnight at 4°C and were then washed in PBS (3 × 5 min) and incubated in a humid chamber with an antimouse IgG-FITC secondary antibody (Dako, Ely, UK, FO232) at a dilution of 1 : 80 for 1 h. Preparations were coverslipped in Vectashield-DAPI mounting medium (Vectorlabs, Peterborough, UK) and analysed using a Nikon Eclipse TC2000-S microscope (Kingston, UK).

FACS analyses

Cells were plated at clonal density and grown for 4–5 days in RM+ medium which was changed to DMEM + 10% FBS for 2 days prior to analysis. In preparation for FACS staining, cells were washed in PBS, and removed from the dish using Accutase® (Chemicon, Chandlers Ford, UK). After rewashing and resuspension at 1×10^6 cells/ml in PBS + 1% FBS, cells were incubated with a CD44-FITC-conjugated antibody (BD Biosciences) at 1 : 100 and/or a CD133/1-PE-conjugated antibody (Miltenyi Biotec, Bisley, UK) at 1:20 for 15 min in the dark (CD29-APC conjugated BD Pharmingen, Oxford, UK, # 559883). The isotype controls used were IgG2ak-FITC (BD Bioscience) and IgG-PE (Abcam). Cells were washed in PBS + 1% FBS, resuspended in 500 µl PBS + 1% FBS, and analysed using a Becton Dickenson LSRII FACS machine (Oxford, UK) and sorted using Becton Dickenson FACSAria equipment. The FITC and PE antibodies were excited using an argon laser (488 nm) and collected using 530/30 nm filter (FITC), 575/26 nm (PE). The APC antibody was excited using the 633 nm red diode and collected at 660/20 nm.

Relationship between colony formation and marker expression

The restriction of clonogenicity to holoclone cells (28) and the greater staining of holoclones with CD44, β1-integrin and, occasionally, with CD133 suggested that cells staining highly for these markers would possess greater clonogenic capacity. To test this assumption, populations of cells expressing high or low levels of these markers were isolated by FACS sorting. Prior to sorting, cells were stained with propidium iodide, to exclude dead cells, and then the 5% of cells with the highest and the lowest expression of CD44, CD29 or CD133, were sorted and were replated at clonal densities. After growth for 5–7 days, cultures were analysed for morphological differences between the colonies that developed.

Side population analyses

The HNSCC cell lines C1, CA1 and UK1 were plated at clonal densities in T75 flasks, harvested as they approached confluence, and resuspended in RM+ medium at 1×10^6 cells/ml. Assays were performed essentially as previously described (37). Aliquots of cells were allowed to equilibrate at 37°C before the addition of Hoechst 33342 to final concentrations of 2.5, 5 and 10 µg. The cells were then maintained at 37°C and samples taken at 15 min intervals up to 1.5 h and stored on ice. Propidium iodide (2 µg/ml) was added for discrimination of dead cells before specimens were analysed. The Hoechst dye was excited by violet laser (407 nm) using FACSAria equipment. Each assay was also run with the addition of reserpine (5 µg/ml) to block ABC transporter function as a negative control.

Sphere formation

To test their ability for growth and sphere formation in suspension, cells were trypsinized, passed through a 40 µm filter to provide a single cell suspension, and 10 ml of medium containing 2×10^4 cells was added to 10 cm dishes pre-coated with PolyHEME (2-hydroxyethyl methacrylate; Sigma, Gillingham, UK; 38). After 1 week plates were visually assayed for the formation of floating spheres. To assess the ability of primary spheres to form secondary spheres, they were collected by centrifugation and digested to single cell suspensions using Accumax (Chemicon). After passing through a 40 µm filter, 10 ml of medium containing 2×10^4 cells was added to Polyheme-coated dishes and assayed after 1 week for secondary sphere formation. To determine the viability and self-renewal abilities of their constituent cells, secondary spheres were collected and passaged into adherent plates to assess their patterns of colony formation.

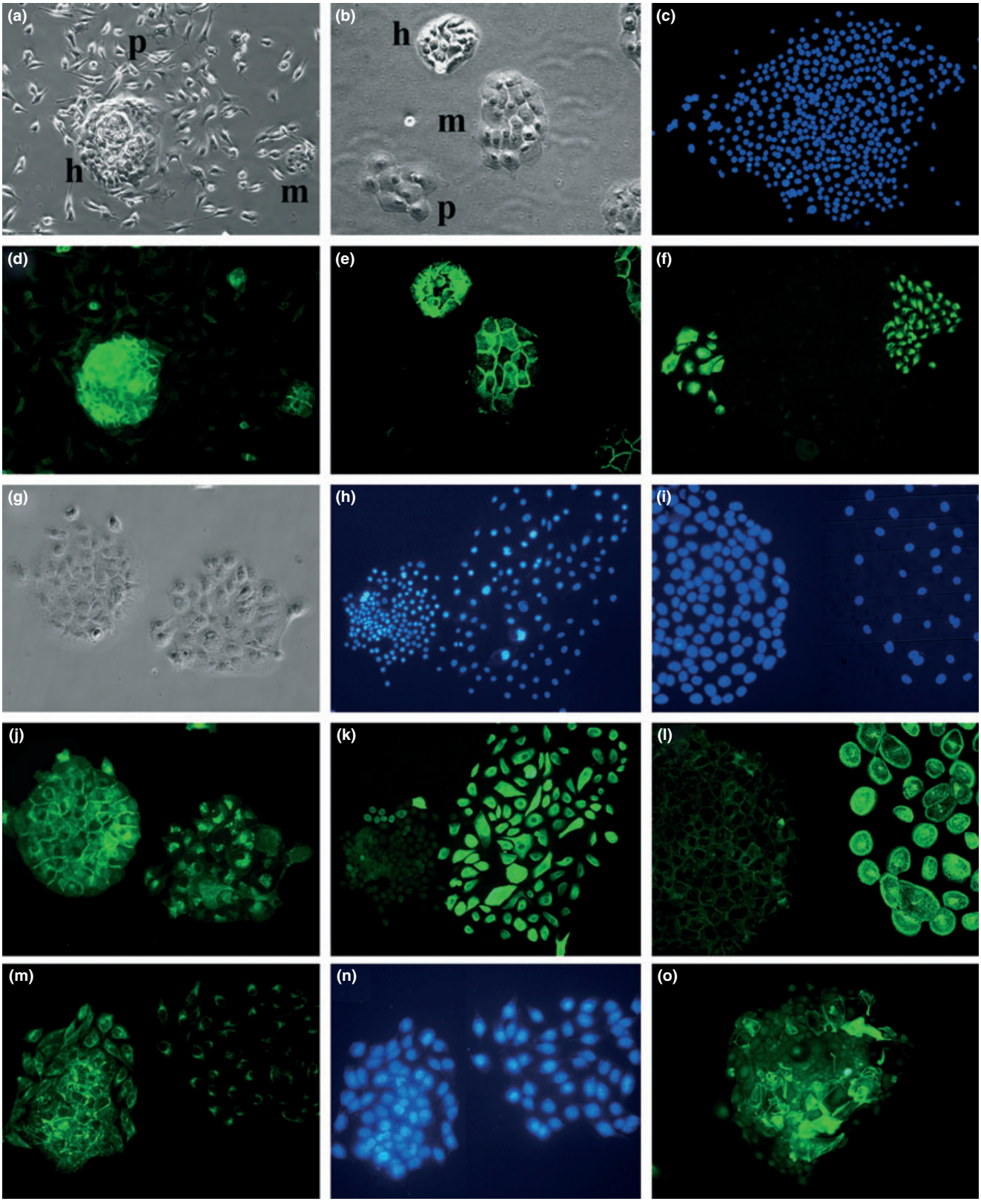
Results

Derivation of cell lines and maintenance of stem cell patterns

Using the method outlined above, cells lines were successfully isolated from six independent HNSCC samples. After initial passage on feeder cells, each of

Figure 1 Relationship of staining patterns to colony morphologies. (a and b) Illustrations of variation in cell and colony morphologies in different cell lines. (a) Phase contrast image of CaLH2 cell line which shows a dispersal of cells as they mature. A colony with holoclone characteristics of circular outline and tightly packed 'cobblestone' cells (h) is surrounded by cells with a spaced and fusiform paraclone morphology (p). A small colony (m) perhaps corresponds to a meroclone. (b) The CaLH3 cell line shows a more cohesive phenotype. The upper colony (h) has holoclone morphology similar to that in (a), the central colony (m) has meroclone morphology with larger irregularly sized cells, and the lower colony (p) has the paraclone features of large flattened cells. (d and e) The same fields stained for β-catenin (d) and CD44 (e) showing strong reactivity of the surface region of holoclone cells, some surface reactivity of meroclone cells, and no reactivity of paraclone cells. (c and f) A large meroclone of the VB6 cell line stained with Hoechst to show nuclear details (c) and for vimentin (f). Cells at the margins of the colony, characterized by increased spacing and larger nuclei, have acquired expression of vimentin. Phase contrast image (g) of a holoclone (left) and meroclone of the H357 cell line and the same field stained for β1-integrin (j) showing cell surface staining of the holoclone and reduced, and mainly cytoplasmic, staining of the meroclone. Holoclone and paraclone colonies of the 5PT line stained with Hoechst (h) and for K14 (k) showing expression of K14 largely restricted to paraclone cells. Holoclone and paraclone cells of the CA1 cell line stained with Hoechst (i) and for K6 (l) with expression of K6 similarly restricted to paraclone cells. Phase contrast image of holoclone (left) and meroclone colonies of the H314 cell line stained for CD133 (m) and with Hoechst (n) showing some surface reactivity of cell in the central region of the holoclone. Paraclone colony of the H357 cell line (o) shows staining of larger flattened cells for K16 indicating their entry into differentiation.

the newly isolated cell lines was able to grow without feeder cells and could be recloned and expanded from single cells. After plating at clonal density, all cell lines displayed a range of heterogeneous colony morphologies consistent with patterns previously published for existing HNSCC cell lines (28). Holoclones were iden-



tified as round colonies consisting of tightly packed small cells, paraclones as colonies of scattered, larger cells with a spindle-shaped or flattened appearance, and meroclones as colonies with intermediate features (Fig. 1). Cell lines showed variation in their rates of growth and also in the details of their colony morphologies, particularly in the degree of scatter and increase of cell size in paraclones. However, for all new and pre-existing cell lines, the three basic colony types persisted through extensive re-passaging.

Expression of stem cell markers in malignant cell lines

Immunocytochemical investigation of two of the newly isolated cell lines (CaLH2 and CaLH3) was used to determine their patterns of expression of molecules reported to act as markers of stem cells or of cell differentiation and to see how these patterns compared with those for the pre-existing cell lines. Examples of the expression patterns observed are illustrated in Fig. 1. The CaLH2 and CaLH3 cell lines, and also the pre-existing cell lines, showed high levels of expression of β-catenin and CD44 restricted to the surface region of holoclone cells. Lower levels of expression of these markers were found for meroclones but paraclones showed little or no expression of either marker. β1-Integrin also showed higher expression at the cell surface region of holoclone cells. For these markers, the patterns of expression were consistent and similar for each of the cell lines examined. Other markers, however, showed a greater degree of variation between individual cell lines. Staining for CD133 was weak and inconsistent but, when present, was found at the surface of cells in central regions of holoclones. Typically, cells of all colony types stained for CK14 but in several cell lines there was less staining of holoclones. A similar pattern was seen for the differentiation marker CK6 which in some cell lines stained all cells but in others was strongly expressed in paraclones but absent from holoclones. Staining for vimentin was variable. It tended to be more strongly expressed in paraclones but in some meroclone colonies, staining for vimentin was also seen in peripheral cells that had acquired the paraclone-like features of larger nuclear size and increased cell spacing (Fig. 1).

FACS analysis of potential stem cell markers

Figure 2 shows the results of FACS analysis of CD44, CD133 and CD29 (β1-integrin) expression in cells of the CA1, H357, CaLH2 and CaLH3 lines. Isotype control antibodies were used to identify the negative population and set gates for CD133. For CD44 and CD29, however, although there was gradient of positivity all cells were positive and control antibodies were used only to assess specificity. High expressing subpopulations were identified using two-dimensional dot plots and side scatter. From this, the discontinuous population at the far right of the plot showing the highest expression was gated. The different cells lines expressed different levels of markers and this assessment was carried out individually for each cell line and marker. Isotype-specific control antibodies were used to identify negative populations and assess the gate

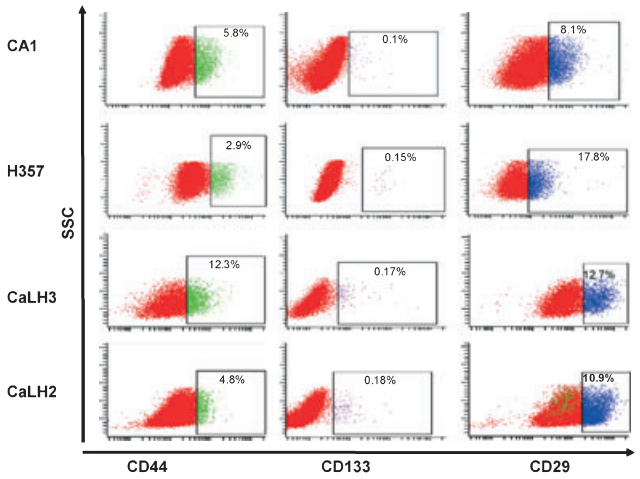


Figure 2 Plots for FACS analysis of cell distribution in the CA1, H357, CaLH2 and CaLH3 cell lines after staining for CD44, CD133 and CD29. Isotype control antibodies (not shown) were used to identify the negative population and set gates for CD133. However, for CD44 and CD29 where all the cells were positive, this was not used and the high expressing subpopulation was identified using two dimensional dot plots for staining combined with side scatter. From this often discontinuous population cells showing highest expression at the far right of the plot were gated. This was performed individually for each cell line and marker.

positions for positive populations. Compared with isotype controls, FACS plots for CD44 and CD29-stained cells showed cells with fluorescence several units higher on a log scale. They often showed an identifiably separate cluster of more highly expressing cells which assisted the positioning of gates but for some populations of stained cells clearly separated populations were not apparent and gates defining high expressing subpopulations were selected more arbitrarily according to the overall pattern of positive and negative staining. Staining for CD133 was less intense but a small fraction of cells with stronger staining could usually be identified. All cell lines expressed CD44 with the proportion of the highly expressing subpopulation for each line (estimated from three separate assays) to range from 2.9 ± 1.1% for the H357 line to12.3 ± 4.7% for CaLH3. The fraction of high expressing CD133 subpopulations was estimated to be lower in all cell lines with only 0.10 ± 0.07% (CA1) to 0.18 ± 0.04% (CaLH2) of cells assessed to show high expression of CD133. A large fraction of cells showed high CD29 expression in a subpopulation ranging from 8.1 ± 0.26% (CA1) to 17.8 ± 0.87% (H357) with intermediate expression levels for CaLH2 and CaLH3. Analysis of double staining for these markers (Fig. 3) indicated that small fractions of the total population stained for both CD44 and CD133 (0.1%), or for CD44 and CD29 (0.8–3.6%). Surprisingly, however, given the wide distribution of CD29 expressing cells, a negligible number of cells was stained for both CD133 and CD29. The general pattern of expression of these three markers was consistent for each the HNSCC cells lines tested.

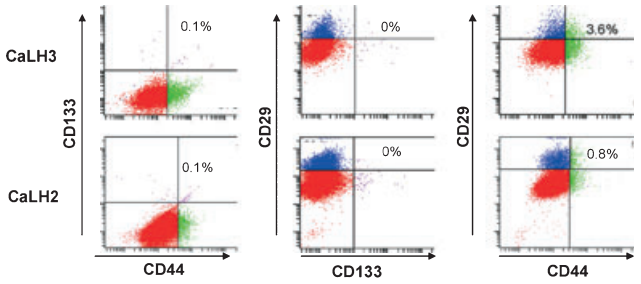


Figure 3 Plots for FACS analysis of cell distribution in the CaLH2 and CaLH3 cell lines showing the patterns of co-expression of staining for CD44, CD133 and CD29.

Relationship of colony formation to marker expression
Figure 4 illustrates the colony patterns formed by populations of the CaLH2 and CaLH3 cell lines sorted by marker expression. For both cell lines, cells sorted for high CD44 expression formed colonies of tightly packed small cells, a typical holoclone morphology, whereas colonies formed by cells with low CD44 expression consisted of larger and more elongated and scattered cells. Cells sorted for high expression of CD133 showed similar patterns of clonogenicity but sorting for CD29 was less predictive. This relationship between clonogenicity and marker expression also for the pre-existing cell lines.

Sphere formation in suspension
Each of the cell lines examined formed floating clusters of cells within 1 week of passaging cells into non-adherent plates. Cell lines showed small but consistent differences in the size of the spheres formed and their patterns of cell packing. Primary sphere formation by the CA1, H357 and CaLH2 cell lines is illustrated in Fig. 5. When primary spheres were dissociated and re-passaged, spheres of all lines showed an ability to form secondary spheres with an increase in the total number of spheres. When cells from secondary spheres were dissociated and plated into standard tissue culture plates, they adhered and formed colonies with mainly holoclone morphologies.

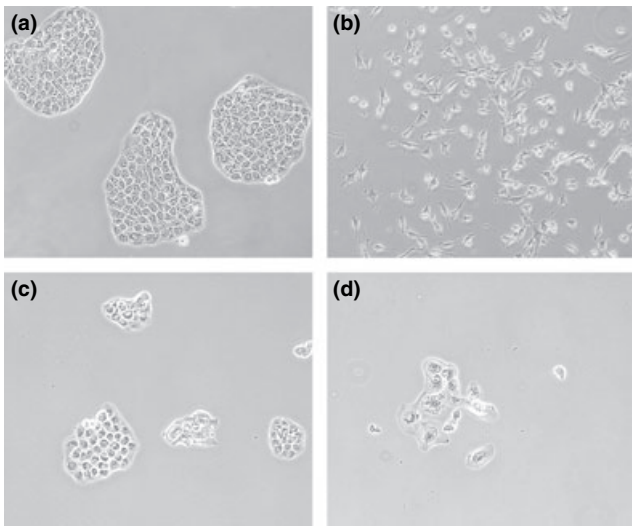


Figure 4 Phase contrast images of colonies developed from cells plated after sorting the 5% of cells with the highest and lowest CD44 expression. The CaLH2 line shows the formation of holoclone/meroclone colonies by cells strongly expressing CD44 (a) and formation of cells with paraclone morphologies by the CD44 low fraction (compare with Fig. 1a). A similar pattern is seen for cells of the CaLH3 line with high (c) and low (d) expression of CD44 (compare with Fig. 1b).

Identification of a side population in malignant cell lines
Cells grown at clonal density were analysed for their ability to differentially efflux the Hoechst 33342 dye. Figure 6 shows the distribution patterns obtained for cells of the Ca1, CH357 and the UK1 cell lines. Cell lines varied quite widely in the timing of dye exclusion but for each line cell analysis showed a subpopulation identifiable by Hoechst dye exclusion and the absence of this subpopulation from cells treated with the membrane pump inhibitor reserpine.

Discussion

The presence of a small subgroup of 'tumour-initiating' cells within myeloid leukaemia was confirmed over 10 years ago (11, 19) and more recent studies have demonstrated the presence of such cells in malignant lesions of brain, breast, colon and HNSCC (5, 24, 27, 30, 35, 39, 40). Morphological heterogeneity is typical of malignant cell lines and has often been attributed to genetic instability and clonal evolution (41). Others have suggested an underlying stem cell pattern (18) and stem cell-related patterns have since been shown to persist in a wide range of cell lines derived from such lesions (26, 42). By showing that clonogenicity is restricted to a subpopulation of the total cells each of which can generate populations with the full range of cellular heterogeneity present in the parent line, we have previously demonstrated that heterogeneity of cells within HNSCC cell lines reflects a stem cell pattern (28). Clonogenicity was consistently associated with particular cellular morphologies and expression patterns, indicating that most cell and colony heterogeneity was due to the presence of hierarchies of cells at different stages of maturation and with varying abilities for self-renewal. The newly isolated cell lines examined in the present study showed clonal characteristics essentially similar to those previously demonstrated for established cell lines. The newly isolated lines also showed consistently stronger holoclone staining for CD44, E-cadherin, β1-integrin, as previously shown for existing HNSCC cell lines (28). There has been controversy as to whether such cells represent 'true' stem cells but it is apparent that a small fraction of the total proliferating cells has the clonogenic properties expected of stem cells and generates the bulk of the tumour mass which consists of cells with amplifying and differentiating characteristics (29). Based on stem cell behaviour in normal epithelia, we have argued that three criteria are sufficient to indicate persistence of a stem cell pattern *in vitro*: (i) the presence of cells with the capacity for extensive self-renewal, (ii) their generation of an amplification hierarchy and

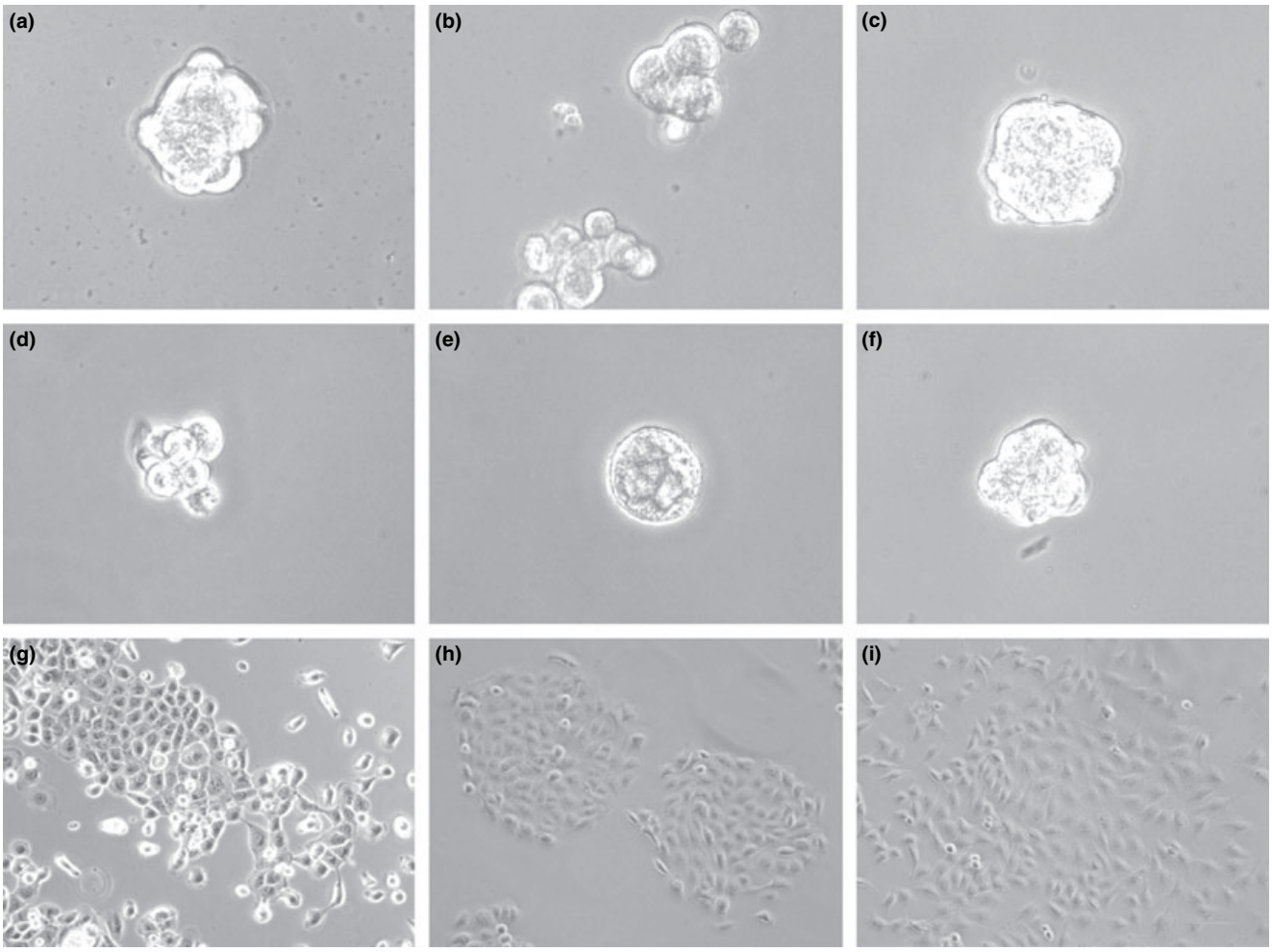


Figure 5 Phase contrast images of primary spheres formed in suspension by the CA1 (a), H357 (b) and CaLH2 (c) cell lines. Panel (d–f) shows secondary spheres for each of these lines, and (g–i) the colonies formed by replating secondary spheres on adhesive dishes.

(iii) production of cells entering a differentiation pathway (28). Several observations indicate that cancer cell lines fulfil these criteria. For example, the expansive growth of malignant lesions indicates the presence of cells with the stem cell property of indefinite self-renewal. Staining of colonies for differentiation markers such as K6 and K16, together with expansion of the stem cell population with passage, indicates the persistence of asymmetric cell fates together with enhanced stem cell self-renewal. The exact degree of correspondence between the clonogenic cells of cell lines *in vitro* and the 'tumour-initiating cells' identified by transplantation *in vivo* remains to be elucidated but considerable overlap of these subpopulations seems likely.

Additional characteristics of malignant stem cells, such as their expression of certain markers, their exclusion of dyes and their growth in suspension, have now been described for gliomas and non-oral carcinomas. One objective of the present study was to determine whether cells with such properties are also present in long-established and newly derived HNSCC cell lines. The stem cell fraction of gliomas, and of prostate and colon cancers, is marked by expression of CD133 (24, 30, 39, 40) but expression of this marker in HNSCC had not previously been reported. Staining intact colonies for CD133 usually produced only weak and inconsistent staining but, when present, staining was restricted to the central region of holoclones, a zone likely to contain stem cells. When cell suspensions were analysed by FACS, which has greater sensitivity, CD133 expression was found for all cell lines and a small population of cells with higher CD133 expression could be identified. Although at relatively low levels, the CD133 expression found in cell lines was consistent with levels previously reported for neuronal and prostate cells (24, 30). However, technically difficulties are associated with preparation of cells for CD133 staining and the epitope recognized by the CD133 antibody is sensitive to proteolytic digestion; preparation of cell suspensions with Accutase, rather than with trypsin, was necessary for consistent staining. Interestingly, although 0.8–3.6% of cells showed co-expression of high levels of CD44 and CD29, only a very small fraction of highly expressing CD133 cells showed high co-expression of CD29 or CD44. How such expression patterns relate to clonogenicity, either *in vitro* or *in vivo*, remains to be determined.

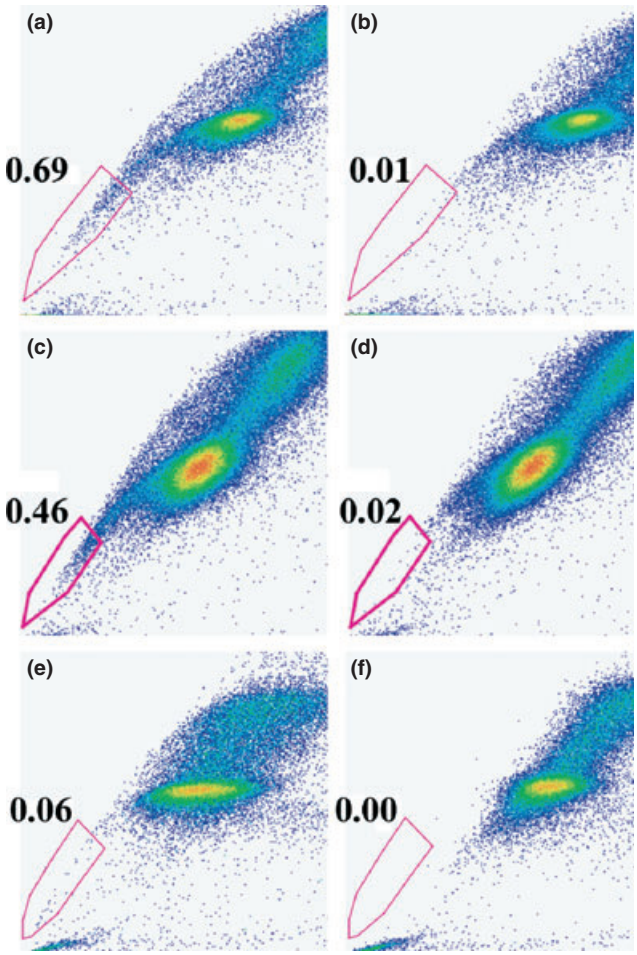


Figure 6 Plots of cell distribution analysing exclusion of Hoechst dye from cells of the UK1 (a and b), CA1 (c and d) and H357 lines (e and f) in the absence (a, c and e) and presence (b, d and f) of reserpine. Each line shows the presence of a population of cells that has excluded dye (gated below and to the left) that is missing when transporter function is inhibited by reserpine. Samples were analysed every 15 min after exposure to the dye and the plots shown are from the time point at which the maximal differences between samples untreated and treated with reserpine were seen. These were at 90, 60 and 30 min, respectively, for UK1, CA1 and C1.

The presence of a 'side population' of cells excluding Hoechst dye has also been taken as an indication of the presence of a stem cell subpopulation in normal and malignant tissues (26, 37) At a dye concentration of 5 μg⁄ml, each of the cell lines examined showed distinct side populations but the incubation times needed to obtain clear separation of this population varied markedly, ranging from 30 to 90 min. However, the characteristics of the populations so identified are not yet entirely clear and the parameters used to isolate side populations are critical (43). Side populations also appear to vary for different tissues and pathological states. For example, although a side population corresponds to a component with stem cell characteristics in haematopoietic, brain, breast and prostate malignancies (26, 42), for normal epidermis there is no correspondence with 'label-retaining cells' or with other properties expected for stem cells (44, 45).

The growth of normal and malignant stem cells in suspension was initially shown for neural cells and subsequently for cells freshly isolated from both normal and malignant breast tissue and for breast cancer cell lines (46). This ability to grow in suspension appears to be a generalized property of malignant stem cells (42) and each of the HNSCC cell lines examined in the present study contained cells with the ability to proliferate in suspension to form 'tumour spheres'. Re-passaging several times at a 1 : 2 dilution ratio resulted in expansion of the number of spheres, suggesting continuing self-renewal of the clonogenic population. When replated onto adhesive dishes cells, cells isolated from secondary spheres plated to form cell populations that were capable of indefinite expansion and developed the range of colony morphologies typical of the parent line. Expansion in suspension therefore did not appear to alter their prior *in vitro* growth characteristics.

For all cell lines, the overall patterns of clonogenicity, markers expression, side population distribution and growth in suspension were essentially similar but some of the details of these patterns differed between cell lines. For example, all lines formed a recognizable range of colonies varying from tightly-packed small cells to loosely-distributed large cells but, as cells differentiated, the degree of increase in cell size and of cell scattering associated with paraclones varied from one cell line to another. Similarly, although all lines showed changes in cell shape with differentiation, some became greatly flattened and others acquired a more spindle shape. Staining for CD44, CD133 and CD29 was found in all cell lines but the proportion of cells strongly expressing these markers differed from one cell line to the next. All cell lines contained a fraction of cells able to export Hoechst dye but the rate of removal varied markedly from one cell line to the next. Similarly, all cell lines formed 'cancer spheres' when grown in suspension but there was variation in the size of spheres and their patterns of cell packing. Such minor differences between cell lines are probably reflective of their random acquisition of different genetic changes during their progression to malignancy but these differences do not seem to be associated with major changes in mechanisms controlling basic stem cell behaviour.

The finding of stem and amplifying patterns in cell lines derived from HNSCC adds support to the concept that there are stem cell patterns in the tumours of origin (5) and this concept has marked clinical implications. For example, if cells with very different properties are continuously generated within tumours, differential responses to radiation and chemotherapeutic strategies are likely to occur within this heterogeneous population. Consequently, therapeutic strategies need to be tested for their fatal actions on the stem cell subpopulations of tumours to ensure clinically effectiveness (6–8, 21, 47). Good assays for assessing *in vivo* differences in the responses of stem and amplifying cells to therapeutic procedures have yet to be developed but *in vitro* assays could be of some value in this process. However, the degree of normality of the behaviour of stem cells in malignant cell lines has yet to be assessed. The isolation

and prolonged culture of malignant cells is likely to be associated with adaptive changes to *in vitro* conditions and cells in standard cultures also lack stromal interactions that may influence malignant cell behaviour (48). However, despite a role of 'niches' in determining some aspects of epithelial stem cell behaviour (49, 50), stem cell division patterns appear largely intrinsic to the epithelium itself and persist, for example, when normal epithelial cells are isolated and grown *in vitro* (34). Further, the stem cell fractions of HNSCC cell lines consistently show patterns of colony formation, self-renewal and macromolecular expression (28) that parallel those found in normal epithelial cultures where clonal morphologies are related to the behavioural properties of stem and amplifying cells (33). The degree to which the stem cell fractions of cell lines actually model the behaviour of malignant stem cells *in vivo* needs to be established but it seems that the persistence of stem cell patterns in malignant cell lines can at least provide an initial model with which to investigate the effectiveness of particular therapeutic strategies against malignant stem cells.

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