Retention of Intrinsic Stem Cell Hierarchies in Carcinoma-Derived Cell Lines

Matthew Locke, Matthew Heywood, Stuart Fawell, and Ian C. Mackenzie

Institute for Cell and Molecular Science, Queen Mary University of London, Whitechapel, London, United Kingdom

Abstract
Recent work indicates that the growth and behavior of cancers are ultimately determined by a small subpopulation of malignant stem cells and that information about the properties of these cells is urgently needed to enable their targeting for therapeutic elimination. A key feature of normal stem cells is their asymmetrical division, the mechanism that allows stem cell self-renewal while producing hierarchies of amplifying and differentiating cells that form the bulk of the tissue. Most cancer deaths result from epithelial malignancies, but the extent to which the hierarchical proliferative stem and amplifying cell patterns of normal epithelia are actually retained in epithelial malignancies has been unclear. Here we show that even cell lines generated from carcinomas consistently produce in vitro colony patterns unexpectedly similar to those produced by the stem and amplifying cells of normal epithelia. From the differing types of colony morphologies formed, it is possible to predict both the growth potential of their constituent cells and their patterns of macromolecular expression. Maintenance of a subpopulation of stem cells during passage of cell lines indicates that the key stem cell property of asymmetrical division persists but is shifted towards enhanced stem cell self-renewal. The presence of malignant epithelial stem cells in vivo has been shown by serial transplantation of primary cancer cells and the present observations indicate that stem cell patterns are robust and persist even in cell lines. An understanding of this behavior should facilitate studies directed towards the molecular or pharmacologic manipulation of malignant stem cell survival.

Introduction
Normal tissue renewal depends ultimately on a subpopulation of cells (termed stem cells) that are characterized by an extensive proliferative potential (1). Stem cells are precisely distributed in vivo in relation to units of epithelial structure (2) and epithelial stem cells typically divide both to renew themselves and to generate transit amplifying cells that undergo a series of amplifying divisions as they differentiate (2, 3). Stem cell homeostasis results from a balanced pattern of asymmetrical division that generates one cell that remains a stem cell and one that becomes committed to differentiation (3–5). The concept that cancers contain a similar subpopulation of malignant stem cells was proposed several years ago (6) and the expansive growth of malignant lesions indicates the presence of cells with at least the stem cell property of indefinite proliferation. However, the nature of stem cell patterns in malignancy remained a matter of controversy (7, 8) until it was shown that human leukemias retain a hierarchical proliferative pattern and that only a small "tumor-initiating" subpopulation, prospectively identifiable by its CD34+, CD38− phenotype, is able to reinitiate tumor growth after transplantation to immune-deficient mice (4, 9). A similar stem cell pattern has since been shown for breast cancers in which the tumor-initiating cells can be prospectively identified by their CD44+, ESA+, CD24+/low, Lineage− phenotype (10).

Morphologic heterogeneity is a typical feature of malignant cell lines and has usually been attributed to genetic instability and clonal evolution (11). The presence of such cellular heterogeneity, together with uncertainty about the effects of cell adaptation to in vitro conditions (12), has cast doubt on the value of using malignant cell lines rather than freshly isolated tumor cells for malignant stem cell studies (13). However, analysis of stem cell properties in vivo is extremely difficult and elucidation of control mechanisms for key properties, such as asymmetrical division (8), would be greatly simplified if they could be studied in in vitro systems (14). Several findings now suggest that malignant cell lines may in fact retain stem cell patterns of behavior. For example, malignant cell lines derived from nonepithelial tumors show behavioral heterogeneity and contain subpopulations of cells with putative stem cell characteristics such as dye exclusion (15–18). For malignant epithelial lines, the isolation of behaviorally different subpopulations, by density sedimentation (19) and by dye exclusion (16, 17), similarly points to the presence of cells with a range of differing proliferative capabilities. Clonogenic assays of malignant epithelial cells in "organotypic" cultures also suggest the presence of cells with differing clonogenic potentials (20).

Despite a role of "niches" in determining various aspects of epithelial stem cell behavior (21), some control of stem cell division patterns is intrinsic to the epithelium itself and persists after cells are isolated and grown in vitro (5). However, when considering the morphologic heterogeneity displayed by malignant epithelial cells, little attention seems to have been given to the fact that even normal keratinocytes are heterogeneous. When plated at low density, normal keratinocytes generate a range of different in vitro colony forms, classified morphologically as holoclones, meroclones, and paraclones, that are derived from, and contain, cells corresponding to stem and early and late amplifying cells, respectively (22). Holoclones take the form of compact round colonies, paraclones form loose irregular colonies, and meroclones have intermediate features. The observation that malignant cell lines often produce a similar range of colony forms prompted us to undertake a more detailed study of their patterns of colony formation. In so doing, we have been able to show that malignant cells have clonogenic properties closely paralleling those of normal epithelial cells and that the cells of malignant holoclones are, like normal holoclone cells, smaller than the cells of meroclones or...
paraclines, more highly clonogenic, and more rapidly adherent to a range of substrates (23, 24). The different colony morphologies displayed within malignant cell lines were found to be associated with different patterns of macromolecular synthesis.

Materials and Methods

Cell lines and growth conditions. Most of the cell lines examined were derived from head and neck squamous cell carcinomas (HNSSC). These were the lines CA1 and UK1 (20); UM-5PT (derived by T. Carey, University of Michigan); the CAL27, SCC4, SCC9, SCC15, SCC25, SCC68, and SCC71 lines (obtained from the American Type Culture Collection); the H314, H400, and H357 lines (25); and the C1 and VB6 subclones that were derived from the H357 line (26). Before experimental use, the cell lines were cloned, and were then passaged several times. Unless otherwise stated, cell culture supplies were bought from Invitrogen Life Sciences.1

Colonies were cloned, and were then passaged several times. Unless otherwise stated, cell culture supplies were bought from Invitrogen Life Sciences.1 Colony formation and staining patterns of the oral cell lines were examined after growth both in a highly supplemented epithelial growth medium termed FAD (27) and in a basal medium consisting of DMEM supplemented with 10% fetal bovine serum. Normal oral keratinocytes were isolated and grown in FAD medium with 3T3 feeder cells as previously described (27), but all of the malignant lines studied grew without the need for 3T3 feeder cells. Two nonoral cell lines were also studied: the DU145 prostate cell line that was grown in the DMEM plus 10% fetal bovine serum and the MCF7 breast tumor line that was grown in the same medium supplemented with 50 μg/ml insulin.

Colony heterogeneity. For studies of colony morphology and staining patterns, actively growing subconfluent cells were dissociated in trypsin/EDTA, plated in T75 flasks at densities ranging from 50 to 200 cells per cm², and examined by phase contrast microscopy as colonies developed. To test the assumption that colonies derived from low-density plating typically have a clonal origin, some tumor populations were transduced with a retroviral vector (MGIN) expressing the enhanced green fluorescent protein gene (28), mixed with nonexpressing cells of the same line, and plated at low density for examination of the developing colonies by fluorescence microscopy. To determine whether the full range of colony morphologies present in an individual malignant cell line can be generated from a single colony type, the C1, CA1, SCC71, CAL27, and VB6 cell lines were plated at low densities in 90-mm Petri dishes for identification of colonies classified as holoclones (i.e., the putative stem cell colonies predicted to contain cells able both to self-renew and to give rise to amplifying cells). These colonies were isolated with cloning rings, their cells dissociated in trypsin/EDTA and then replated in T75 flasks for analysis of their developing patterns of colony morphology monitored both initially and following further passage.

Adhesion assays. Populations of cells characterized by their rates of adhesion were obtained for the CA1, C1, 5PT, UK1, VB6, MCF7, and DU145 cell lines. Early, intermediate, and late-adhering cells were generated by plating cells in T75 flasks until about 10% of the cells were initially adherent, typically 10 to 30 minutes. The medium containing the nonadherent cells was then removed and added to new flasks for 1 hour. The medium and nonadherent cells were again removed and replated for 4 hours. At the end of each plating period, flasks were washed to remove any weakly adherent cells. For each test, two flasks were prepared for each line and after adhesion, the cells were either incubated to observe the colony morphologies that developed or removed (using trypsin/EDTA) for preparation as cell smears for digital imaging and measurements of cell size by Scion Image software.2

Antibodies and immunofluorescent methods. Colonies growing in T75 flasks were fixed for 10 minutes in ice-cold acetone/methanol before removing the flask bases and processing them for immunofluorescent staining as previously described (27). The antibodies used were mouse monoclonal antibodies against CD29 (Upstate Biotechnology, Lake Placid, NY); β-catenin (BD Biosciences, San Jose, CA); epithelial growth factor receptor (Neomarkers, Fremont, CA); E-cadherin (Zymed, South San Francisco, CA); CD44 (PharMingen, San Diego, CA); epithelial-specific antigen (Novocastra, Clone VU-1D9, Newcastle-upon-Tyne, United Kingdom); and cytokeratins 5, 6, 14, and 16 (mAbs AE14, LL020, LL001, and LL025, respectively, gifts from Prof. Irene Leigh, I.C.M.S., Queen Mary, University of London, London, United Kingdom). Cytokeratin 15 was detected with a chicken polyclonal antibody, a gift from Dr. G. Cotsarelis (M8 Stellar-Chance Laboratories, Philadelphia, PA; ref. 2). The second layer antibodies used were FITC-conjugated rabbit anti-mouse (DAKO, Carpenteria, CA) and rabbit anti-chicken (Sigma, St. Louis, MO) immunoglobulins. Controls for specificity included omission of primary antibodies and comparison of staining with irrelevant antibodies of a range of isotypes. Cells were counterstained with Hoechst 33258 (1 μg/ml) for visualization of nuclear morphology and samples were viewed on an Olympus Provis AX70 fluorescent microscope and photographed using a Nikon DXM1200 digital camera. Separate images for FITC and Hoechst were obtained for each field and combined in Adobe Photoshop 6 when helpful to examine cell distribution.

Cell proliferation. To label cells in the S phase of the cell cycle, 5-bromo-2-deoxyuridine (BrdUrd, Sigma) was added to culture medium to a final concentration of 50 μg/ml. After 2 hours of exposure, cells were fixed in 70% ethanol for 10 minutes, washed in PBS, treated with 4 mol/L hydrochloric acid for 10 minutes, and then washed sequentially in PBS at pH 8.5 and 7.4. Before application of an anti-BrdUrd primary antibody (DAKO, Clone Bu20a) at a dilution of 1:100, endogenous peroxidase was blocked by treatment for 5 minutes with 3% hydrogen peroxide in PBS containing 0.2% Triton 100-X.2 After overnight incubation at 4°C, cells were washed and rabbit anti-mouse secondary antibody conjugated to horse-radish peroxidase applied at a dilution of 1:200 for 2 hours before visualization of binding using a 3,3′-diaminobenzidine substrate kit (Vectastain ABC kit, Vector Laboratories, Burlingame, CA).

RNA extraction and quantitative PCR. A pilot examination of holoclines and paracline RNA extracts, using Affymetrix HG-U133A Arrays, provided preliminary gene expression data for holoclones and paraclines of the C1 and CA1 cell lines and 10 differentially regulated genes (listed in Table 1) were selected for further examination. Material for analysis by quantitative PCR was generated from large holoclone and paracline colonies of seven cell lines (listed on Table 1) that were isolated with cloning rings, replated into T75 flasks, and grown for 2 days to provide expanded cell populations. Total RNA was extracted using an RNAwiz (Ambion, Austin, TX) kit, first-strand synthesis from 1 μg of total RNA was achieved using a Bio-Rad iScript cDNA synthesis kit, both according to the manufacturer's instructions, and cDNA was quantified using a GeneSpec 1 spectrophotometer (Naka Instruments, Kyoto, Japan). Thermal cycling conditions and primer specificity were initially determined by PCR and the subsequent quantitative PCR was done using equal quantities of holoclone or paracline cDNA and a DyNaNo SYBR green qPCR kit (Finnzymes, Espoo, Finland). All assays were repeated at least in triplicate, and the specificity of amplification for all primers (Table 1) was determined from thermal cycler generated melting curves.

Cells derived from holoclones of seven oral cell lines (CA1, C1, VB6, VH-26, HNSCC) were plated singly in 24-well plates, a high proportion of isolated cells showed cell morphology and colony proportions characteristic of the original parental lines. With two or three further passages, the particular patterns of cell morphology and colony proportions of these oral lines, failure of paraclone cells to form colonies was not due to failure of plating or lack of initial proliferative activity; most wells showed formation of aborted microcolonies with the appearance of late paraclones (Fig. 2B). Colony morphology and clonogenic differences between their constituent cells (Fig. 2A) were similarly predictive of similar patterns of colony morphology and clonogenicity, two long-established cell lines, MCF7 and DU145, derived from adenocarcinoma of breast and prostate, respectively, were examined. Plated at low densities, both of these lines developed patterns of cell and colony morphologies corresponding to holoclones and paraclones (Fig. 1D) and these morphologies were similarly predictive of clonogenic differences between their constituent cells (Fig. 2B). Microscopic examination of the plated wells indicated that, as for oral lines, failure of paraclone cells to form colonies was not due to failure of plating or lack of initial proliferative activity; most wells showed formation of aborted microcolonies with the appearance of late paraclones (Fig. 2C). These differed markedly from the colonies formed by holoclone cells that consisted mainly of small individual differences but, superimposed on these, each line showed a spectrum of colony morphologies ranging from round colonies of small closely packed cells to irregularly shaped colonies. Similar patterns of colony morphology and clonogenicity, two long-established cell lines, MCF7 and DU145, derived from adenocarcinoma of breast and prostate, respectively, were examined. Plated at low densities, both of these lines developed patterns of cell and colony morphologies corresponding to holoclones and paraclones (Fig. 1D) and these morphologies were similarly predictive of clonogenic differences between their constituent cells (Fig. 2B). Microscopic examination of the plated wells indicated that, as for oral lines, failure of paraclone cells to form colonies was not due to failure of plating or lack of initial proliferative activity; most wells showed formation of aborted microcolonies with the appearance of late paraclones (Fig. 2C). These differed markedly from the colonies formed by holoclone cells that consisted mainly of small packed cells that could be removed and replated to generate new sublines. The early presence of mixed colonies in some of the wells plated with a single holoclone cell provided further evidence of the ability of holoclone cells to give rise rapidly to all clonal morphologies.

### Results

**Colony morphologies.** After plating at low densities, malignant cells formed small colonies within 2 to 3 days (Fig. 1A). Examination of the colonies formed by mixtures of GFP+ and GFP− cells showed that the great majority of colonies consisted only of one or other cell type and had therefore most probably developed clonally (Fig. 1B). The patterns of cell and colony morphologies developed by the 15 HNSCC-derived malignant cell lines initially examined showed small individual differences but, superimposed on these, each line showed a spectrum of colony morphologies ranging from round colonies of small closely packed cells to irregularly shaped colonies of late paraclones (Fig. 2C). These differed markedly from the colonies formed by holoclone cells that consisted mainly of small packed cells that could be removed and replated to generate new sublines. The early presence of mixed colonies in some of the wells plated with a single holoclone cell provided further evidence of the ability of holoclone cells to give rise rapidly to all clonal morphologies.

**Colonies on passage.** After three further passages, the particular patterns of cell morphology and colony proportions characteristic of the original parental lines were restored indicating that the cells of malignant holoclones, like those of normal keratinocyte holoclones, are able to regenerate the normal keratinocyte phenotype on passage. With two or three further passages, the particular patterns of cell morphology and colony proportions characteristic of the original parental lines were restored indicating that the cells of malignant holoclones, like those of normal keratinocyte holoclones, are able to regenerate the normal keratinocyte phenotype on passage.

### Table 1. Assessment by q-PCR of differences in gene expression between holoclones and paraclones for each of seven cancer cell lines

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>C1</th>
<th>CA1</th>
<th>VB6</th>
<th>SCC71</th>
<th>CAL27</th>
<th>MCF7</th>
<th>DU145</th>
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<td>Notch 3</td>
<td>Forward TGTGGACGAGTGCCTCTATCG</td>
<td>16.7</td>
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<td>42</td>
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<td></td>
<td>Reverse AATGTCACCTCCTGAATAGG</td>
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<tr>
<td>IGF3BP3</td>
<td>Forward CCTGCGTAGAGAAATTGGA</td>
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<td>4.9</td>
<td>4.7</td>
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<td>0.1</td>
<td>7.4</td>
<td>2.7</td>
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<tr>
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<tr>
<td>Hur7</td>
<td>Forward TGACATCTGGAAACCTGTG</td>
<td>2.8</td>
<td>4</td>
<td>3</td>
<td>17.1</td>
<td>5.2</td>
<td>12.1</td>
<td>3.6</td>
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<tr>
<td></td>
<td>Reverse TAAACTCTGCTGCCATCTTG</td>
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<td>CEBP</td>
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<td>4.6</td>
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<td>4.1</td>
<td>1</td>
<td>1.6</td>
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<tr>
<td>Pirin</td>
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<td>14.7</td>
<td>5.2</td>
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<td>3.2</td>
<td>8</td>
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<td>Reverse GCCCTTTCCAAACCATTTT</td>
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<td>RDHL</td>
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<td>29.8</td>
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<td>4.9</td>
<td>4.6</td>
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<td></td>
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<td>PSCA</td>
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<td>2.1</td>
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<td>Erb-B3</td>
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<td>13.6</td>
<td>3.1</td>
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<tr>
<td>HPGD</td>
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<td>4.6</td>
<td>10.2</td>
<td>11.9</td>
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<tr>
<td>E-CAD</td>
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Note: >1, fold higher expression in holoclones; <1, lower expression in holoclones. Full gene names (accession nos.) are Notch 3 (gb:NM_004351.1); IGF3BP3, insulin-like growth factor binding protein 3 (gb:BF340228); Hur7, UV-B-repressed hurpin 7 (gb:BE148534); CEBP, CCAAT enhancer-binding protein α (gb:NM_004364.1); Pirin, v-erb-b2 avian erythroblast leukemia viral oncogene homologue 3 (gb:NM_001982.1); HPGD, hydroxyprostaglandin dehydrogenase (gb:NM_000860.1); and E-CAD, E-cadherin (gb:BF215158).
the more rapidly adherent cells was consistently smaller than that of the less rapidly adherent cells (Fig. 3A). Counts of the types of colony morphologies formed by early, intermediate, and late-adhering cells indicated that the smaller early adherent cells formed a greater proportion of holoclone colonies (ANOVA, \( P = 0.003 \)), indicative of their greater clonogenicity. Staining for BrdUrd incorporation into large colonies indicated that there was little cell proliferation in paraclones that consisted only of greatly flattened cells. However, all other types of colonies, from holoclone to early paraclones, showed a large proportion of cells that had incorporated BrdUrd (Fig. 2D), and these colonies also showed similar rates of increase in size with further time in culture.

Figure 1. Colony morphologies in malignant cell lines. A, range of early colony morphologies formed by the lines CA1, C1, and UK1, respectively. Colony morphology varies from that of holoclones (left) to paraclones (right). Intermediate meroclone forms. The continuous gradient of colony morphologies lacks clear cutoff points for classification, but holoclones are recognized as tight clusters of uniformly small cells producing a smooth outline and paraclones are characterized by loss of tight cell packing, larger cell size, and irregular cell and colony shapes. The cell and colony morphologies vary somewhat from one cell line to another with most differences being apparent for paraclone cells which can be highly flattened (CA1), show multiple filopodia (C1), or be spindle-like (UK1). The range of CA1 colony morphologies was developed at the first plating of cells isolated from a single cell. Twenty-four-well plates in which each well was individually seeded with a single cell derived from either a holoclone or paraclone. Twenty-four-well plates in which each well was individually seeded with a single cell derived from either a holoclone or paraclone. B, phase-contrast and fluorescent images of colonies formed by the singly plated CA1 holoclone cell (top right) show expanding colonies compared to only four wells seeded with paraclone cells. In other similar experiments, it was shown that colonies derived from holoclones were capable of indefinite further passage. C, markedly different microscopic appearance of colonies are formed by holoclone and paraclone cells. Holoclone cells of the DU145 line form large colonies of packed small cells and paraclone cells form colonies with spaced, often spindle-shaped, cells with little growth. The paraclone cell from the MCF7 line shows little growth even 21 days after plating cells. For all cell lines, plating single holoclone cells initially generated colonies with holoclone morphologies but, as shown by the singly plated CA1 holoclone cell (bottom right), meroclones can arise at an early stage, and following further growth, paraclone colonies developed. D, however, two colonies, a holoclone (left) and a paraclone (right), after staining to display cells incorporating BrdUrd. More than half of the holoclone cells are labelled and also quite a high proportion of the early paraclone cells. Bar, 0.3 mm (C) and 0.2 mm (D).

Immunofluorescent staining patterns of oral cell lines. Each of the cell lines examined showed differing patterns of staining for holoclones and paraclones with antibodies against the majority of the markers examined. Cell lines varied in their degree of staining with particular antibodies, but holoclones typically showed stronger staining than paraclones for molecules that have been reported to be expressed more strongly by normal stem cells. For example, in all cell lines, most colonies showed some staining of cell peripheries for \( \beta \)-catenin, but staining of holoclone cells was much brighter than paraclone cells (Fig. 4A). Similarly, all cell lines showed stronger staining of the cell peripheries of holoclone cells for \( \beta \)-catenin (Fig. 4A) and E-cadherin (Fig. 4B). Maintenance of an epithelial phenotype was indicated for all cell lines by staining for cytokeratin 5 (Fig. 4B), and cytokeratin 14, typically basal cell markers in stratifying squamous epithelia. Staining for cytokeratin 15 was absent from the CAL27 and DU145 cell lines and showed only weak cytoplasmic staining for most colonies of other cell lines. However, much stronger cytoplasmic staining of holoclones for K15 was found in the C1 and CA1 (Fig. 4C). Staining for cytokeratins 6 and 16, markers of early cell differentiation in normal oral mucosa, was present in most cell lines with large flattened cells in
paraclines being strongly stained, a pattern typical of normal oral keratinocytes in culture (Fig. 4D).

The clonal heterogeneity present in the MCF7 breast cell line allowed examination of how holoclone morphology, considered predictive of the stem cell phenotype, correlated with the expression of CD44 and ESA, markers used in the isolation of “tumor-initiating cells” from freshly isolated or in vivo passaged breast tumor cells (10). Colonies formed by the MCF7 line showed tight restriction of staining for CD44 to the peripheries of cells in holoclones (Fig. 5A). To examine whether the MCF7 pattern of staining for CD44 was characteristic of other cell lines, colonies formed by the CA1, C1, VB6, and DU145 cell lines were stained for CD44 expression and each line showed a similar pattern of restriction of CD44 staining to cells within holoclones (Fig. 5A-C). Holoclones formed by the MCF7 line also stained strongly for ESA, but staining was less tightly restricted and extended weakly to meroclones. The other cell lines similarly showed stronger staining of holoclones with ESA and extension of decreasing staining to meroclones (Fig. 5D and D). Staining for cytokeratin 15 was present in the MCF7 cell line and was restricted to holoclones (data not shown).

Genic expression patterns assessed by quantitative PCR. Table 1 shows the results of examination of differences in gene expression between holoclone and paraclone colonies of five HNSCC cell lines and the two non-HNSCC cell lines. The data in Table 1 were calculated on the basis of equalized input quantities of cDNA (29). For all of the cell lines, 6 of the 10 genes monitored were found to be expressed at higher levels in holoclone colonies than paraclone colonies. The exceptions were the CAL27 cell line that showed lower expression of IGFBP3 in holoclones and the DU145 line that showed lower holoclone expression of CEBPα, pigrin, and E-cadherin.

Discussion

The primary aims of these investigations were to determine whether the morphologic heterogeneity typically found in malignant epithelial cell lines is mainly the result of an underlying stem and amplifying cell pattern and, if so, how differing cellular properties are predicted by such morphologic differences. Given the often controversial nature of stem cell studies, a problem initially encountered was to define what experimental criteria might reasonably be accepted as indicating the functional presence in malignant cell lines of stem cell patterns similar to those of normal keratinocytes. Three criteria were chosen, based on those for normal epithelia: (a) the presence of a subpopulation of cells with the capacity for extensive self-renewal, (b) their generation of an amplification hierarchy, and (c) their production of cells entering a differentiation pathway (5).

Fulfillment of the first criterion could be inferred from the continuous expansive growth of malignant cell lines, both in vivo and in vitro. In addition, as new lines can be repeatedly generated from singly plated holoclone cells, it is apparent that cells with the property of indefinite self-renewal are present and are able to expand their numbers under in vitro conditions. The clonal assays that were done with malignant cell lines were essentially similar to those used previously for in vitro investigation of stem and amplifying cells from normal epithelia (22). All of the cell lines examined showed marked clonal heterogeneity and developed a range of colony morphologies paralleling the holocline, meroclone, and paraclone morphologies produced by normal keratinocytes. The relatively high rates of cell proliferation in meroclone and early paraclone colonies suggest that they contain the equivalent of early- and late-amplifying cells with holoclone cells differing from paraclone cells in being smaller, more rapidly

Figure 3. A, measurements of the size of early (solid columns), intermediate (cross-hatched columns), and late-adhering cells (dotted columns) of the (A) CA1, (B) C1, (C) SPT, (D) UK1, and (E) VB6 cell lines. For all lines, small cell size correlates with more rapid adhesion. B, percentage of BrdUrd-labeled cells in holoclones (solid columns) and paraclones (cross-hatched columns) of the (A) CA1, (B) C1, (C) SPT, (D) UK1, (E) VB6, (F) MCF7, and (G) DU145 cell lines. Both types of colonies show relatively high rates of proliferation but with holoclones consistently higher than paraclones (ANOVA, P < 0.05).

Figure 4. Immunofluorescent staining of colonies. A, strong cell surface staining of holocline cells of the CA1 and C1 cell lines for β1 integrin and β-catenin but little reactivity of paraclone cells (right). B, a large VB6 holoclone with strong cell surface staining for E-cadherin abuts a large paraclone with reduced staining. The SPT line shows cytoplasmic staining of all cells for cytokeratin 5 but with stronger staining of holoclone cells (left). C, strong staining is seen for cytokeratin 15 in C1 holoclone cells but not of the dispersed cells of the large adjacent paraclone, apparent when the same field is viewed to show staining for Hoechst 333258 (right). D, a population of large cells overlying the centre of a colony of normal oral keratinocytes (left) shows staining for cytokeratin 6 and a similar pattern is seen for the C1 paraclone colony. Bar, 0.15 mm (A and D) and 0.25 mm (B-C).
in malignant holoclones raises the possibility that some markers may be selectively expressed by stem cells in malignant tissues.

Differences between the stem cells of a cell line and the stem cells that were present in its tumor of origin are likely to result both from cellular changes acquired during the generation of the cell line and from the tissue culture environment itself. Thus, although cells acting as malignant stem cells in vitro can be prospectively identified by morphologic and expression characteristics, it remains uncertain how closely they correspond to "tumor-initiating cells" identified by in vivo transplantation of cells freshly isolated from tumors (10). The in vitro colonies generated by malignant cell lines, like those of normal keratinocytes, form a continuous gradient extending from "ideal" holoclone morphology, through meroclones, to early and late paraclones, but where on this morphologic gradient the property of in vitro self-renewal is lost remains to be determined. Observations made during the present work suggest that cell lines differ in the fraction of holoclone colonies they contain, whether these are identified morphologically or by staining for markers such as CD44. Furthermore, the percentage of singly plated holoclone cells found to generate new holoclone colonies varied quite widely between cell lines (range, 47-100%; data not shown). In a previous study (20), we found that the fraction of total cells with in vitro clonogenic abilities also varies between cell lines, and it will be interesting to compare different cell lines for the proportions of cells capable of initiating new cell lines in vitro and the proportion able to act as tumor-initiating cells after in vivo transplantation.

Demonstration of stem cell patterns in hematologic malignancies (9), and more recently in malignancies of epithelial origin (10), has indicated that elimination of the stem cell subpopulation within tumors is a necessary component of effective therapy (4, 13, 38). Although the great majority of cancer deaths are caused by malignancies of epithelial origin (39), less is known about the behavior of epithelial stem cells, whether normal or malignant, than about their hematopoietic equivalents. However, it is known that normal epithelial stem cells differ from amplifying cells in their growth patterns, apoptotic sensitivities, and motilities (40, 41). They also have higher expression of multidrug resistance transporters (42), but how the retention of this and other differential properties in epithelial tumors (15–17) influences the outcomes of therapeutic procedures presently available remains to be determined.

Another point of interest is that normal homeostatic renewal of epithelia depends on a balanced pattern of asymmetrical stem cell division (4, 5) and thus differs from the growth typical of tumors in which stem cell expansion results from a persistent shift towards enhanced self-renewal (4, 43, 44). The changes that underlie altered patterns of stem cell self-renewal, in either normal or malignant tissues, remain uncertain (31, 32, 43, 45), but normal stem cell self-renewal is at least partially responsive to environmental conditions and is, for example, reduced by cell isolation but increased during growth, in wound healing, and under certain in vitro conditions (8, 30). Possibly, therefore, the self-renewal patterns of malignant stem cells could be manipulated to produce tumor atrophy by directing malignant stem cells away from symmetrical division, a modification of the concept of "directed differentiation" previously raised (46). Analyses of molecular mechanisms associated with asymmetrical division should be facilitated by the persistence of asymmetrical division patterns in malignant cell lines. The in vitro differences identified between
holoclone and paracline cells provide a range of potential molecular and metabolic properties for selective therapeutic actions and the readily quantifiable stem and amplifying populations present in malignant cell lines provide a relatively simple in vitro system for the initial screening of therapeutic agents for their actions on malignant stem cells.

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Matthew Locke, Matthew Heywood, Stuart Fawell, et al.


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