Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative

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Running Title

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Abstract

Epithelial-to-mesenchymal transition (EMT) is an important driver of tumor invasion and metastasis, which causes many cancer deaths. Cancer stem cells (CSCs) that maintain and initiate tumors have also been implicated in invasion and metastasis, but whether EMT is an important contributor to CSC function is unclear. In this study, we investigated whether a population of CSCs that have undergone EMT (EMT CSCs) exists in squamous cell carcinoma (SCC). We also determined whether a separate population of CSCs that retain epithelial characteristics (non-EMT CSCs) is also present. Our studies revealed that self-renewing CSCs in SCC include two biologically-distinct phenotypes. One phenotype, termed CD44\textsuperscript{high}ESA\textsuperscript{high}, was proliferative and retained epithelial characteristics (non-EMT CSCs), whereas the other phenotype, termed CD44\textsuperscript{high}ESA\textsuperscript{low}, was migratory and had mesenchymal traits characteristic of EMT CSCs. We found that non-EMT and EMT CSCs could switch their epithelial or mesenchymal traits to reconstitute the cellular heterogeneity which was characteristic of CSCs. However, the ability of EMT CSCs to switch to non-EMT character was restricted to cells that were also ALDH1\textsuperscript{+}, implying that only ALDH1\textsuperscript{+} EMT cells had the ability to seed a new epithelial tumor. Taken together, our findings highlight the identification of two distinct CSC phenotypes and suggest a need to define therapeutic targets that can eradicate both of these variants to achieve effective SCC treatment.
Introduction

Several studies have implicated cancer stem cells (CSCs) in tumour invasion and metastasis (1-2) and have related tumour recurrence after therapy to therapeutic resistance of CSCs (3-4). Typically, CSCs are defined as a subpopulation of tumour cells having both tumour-initiating ability and the ability to reconstitute the cellular heterogeneity typical of the original tumour (5). For solid tumours, CSCs with these properties were first demonstrated in breast cancers as cells with a CD44<sup>high</sup>CD24<sup>low</sup>ESA<sup>+</sup> staining pattern (6). As for several other malignancies, a sub-population of CD44<sup>high</sup> cells with CSC properties has been identified within oral squamous cell carcinoma (OSCC) (7). In breast cancer and OSCC, sub-populations of cells with CSC properties have also been identified by positive activity of the detoxifying enzyme ALDH1 (8-9).

Epithelial-to-mesenchymal transition (EMT) is a developmental process that creates mesoderm during gastrulation, in which epithelial cells acquire a migratory mesenchymal phenotype (10). In adult tissues, several stromal signals including TGFβ can induce EMT and lead to down-regulation of epithelial products such as E-cadherin and upregulation of EMT-inducing transcription factors, such as Twist and Snail (11-12). EMT has been proposed to play important roles in cancer and mutations in receptor tyrosine kinase or Wnt signaling pathways can predispose cells to undergo EMT (13). In cancer there is further promotion of EMT by both the inflammatory immune response (14) and by the hypoxic tumour environment (15). Breast cancer cells with the CD44<sup>high</sup>CD24<sup>low</sup> tumour-initiating phenotype express EMT markers, a finding that established a link between CSCs and EMT (16) and it is now increasingly recognized that EMT plays an important role in the metastasis of OSCC (15), breast cancer (17), and several other types of carcinoma. EMT has been implicated in therapeutic resistance and tumour recurrence (4, 18-19) and is associated with resistance to EGFR inhibitors (20) and evasion of host immune responses to tumours (17, 21).
Normal epithelial tissues are maintained by a proliferative stem cell hierarchy and, when epithelial cells are seeded at low density in vitro, the stem cell fraction generates proliferating colonies termed holoclones that can be distinguished from the abortive colonies of differentiating cells, termed paraclones (22). Similar clonal patterns are present in cell lines generated from OSCC (23) and also from prostate cancers where holoclone cells, but not paraclone cells, are capable of forming tumours in mouse xenograft models (24). Here we demonstrate that, in addition to a holoclone-forming CSC population, cell lines derived from human oral and cutaneous SCC contain a novel CSC population that has undergone EMT and is migratory. The EMT and non-EMT CSC populations both show high expression of CD44 and the two populations co-exist by switching between the two phenotypic states through EMT and the reverse process of mesenchymal-to-epithelial transition (MET). Both cell types are also present in cells freshly generated from OSCC tumours. We also demonstrate a further novel hierarchy within the EMT cell subpopulation itself that is related to the ability of cells to switch back to the non-EMT state.

Materials and Methods

Cell culture

The CA1 cell line was previously derived in our laboratory from a biopsy of OSCC of the floor of the mouth (25). The PM1, Met1 and Met2 cell lines were derived from matched premalignant and malignant cutaneous SCC tissues (26). All cell lines were grown in the highly supplemented epithelial growth medium (termed FAD) with 10% FBS (23). For suspension cultures, 0.75cm² tissue culture wells were coated with polyhema (Sigma) (12mg/ml in 95% ethanol). Cells were then plated at a density of 1000 cells/ml with addition of 1% methylcellulose (Sigma) and incubated (37°C, 5% CO₂).
and monitored for sphere growth. For TGF-β experiments, 20 ng/ml TGF-β (R&D systems) was added to the culture every 24 hours for 5 days. For inhibition, 10 μM SB431542 (Sigma) was added either simultaneously with the TGF-β additions or for 5 days following 5 days of TGF-β treatment.

**FACS and Immunofluorescence**

For FACS, cells were detached using either trypsin-EDTA (PAA) or enzyme-free cell dissociation buffer (Invitrogen) at 37°C and then stained with antibodies at 1:100 dilution in PBS (PAA). The DAPI nuclear dye (Sigma) was used at 1 ug/ml to exclude dead cells. FACS sorted cells were collected into FAD medium for plating or into buffer RLT for RNA extraction. To test for ALDH1 activity, cells were stained with Aldefluor reagent (Aldagen) according to the manufacturers instructions prior to FACS. For immunofluorescence, cells were fixed in 4% paraformaldehyde, stained with antibodies at 1:100 dilution in PBS with 0.25% BSA, then permeabilised with 0.1% Triton-X prior to addition of DAPI at 1 ug/ml prior to imaging. The PE-CD44 (clone G44-26) and PE-Integrin β4 (clone 439-9B) antibodies were from BD biosciences; the APC-ESA (clone HEA-125) antibody was from Miltenyi Biotec.

**RNA extraction, cDNA synthesis and QPCR**

RNA was extracted using the RNeasy micro kit (Qiagen). Reverse transcription into cDNA was conducted using the Superscript III first strand synthesis supermix (Invitrogen), with inclusion of −RT controls. QPCR was conducted in an ABI 7500 real-time PCR system (Applied Biosystems) using Power SYBR green mix (Applied Biosystems). GAPDH was used as a reference mRNA control. See supporting information for QPCR conditions and primer sequences.
**Migration assays**

10,000 cells were placed in FAD medium with 2% FBS in Transwell tissue culture inserts (8um membrane, Corning) in 24-well plates, with FAD medium containing 10% FBS in the bottom of the well. After 24 hours, the membranes were fixed in 4% paraformaldehyde, stained with crystal violet, the non-migrated cells on the top of the membrane removed with a cotton wool bud, and the migrated cells on the underside of the membrane counted.

**Generation of cells from primary human samples**

Fresh OSCC tissue samples were collected following protocols approved by the local NHS Research Ethics Committee. They were minced into small pieces in tissue culture dishes, allowed to adhere, and FAD medium then added. Outgrowths of cells arising from the tumour pieces were dissociated and FACS sorted for the epithelial-specific marker Integrin β4 to collect fibroblast-free populations prior to examination.

**Single cell cloning**

For single cell cloning of the CD44\textsuperscript{high}ESA\textsuperscript{high} and CD44\textsuperscript{high}ESA\textsuperscript{low} populations, cells were sorted by FACS and then allowed to recover in culture before being resuspended, counted, and single cell cloned in 48-well plates by limiting dilution. Wells were examined microscopically and those containing only a single clone were grown up for analysis. For single cell cloning of the CD44\textsuperscript{high}ESA\textsuperscript{low/+} and CD44\textsuperscript{high}ESA\textsuperscript{low/-} populations, cells were sorted directly into 96-well plates using the single cell plate sorting function of the FACSaria FACS sorter (BD biosciences). Clones were then checked and grown up as described above.
Tumourigenesis assays in mice

NOD/SCID mice were used with all animal procedures approved by the Norwegian Animal Research Authority. Subpopulations of FACS-sorted CD44<sup>high</sup>ESA<sup>high</sup> and CD44<sup>high</sup>ESA<sup>low</sup> CA1 cells were suspended in 50μl of Matrigel (BD Biosciences) and transplanted into the tongue. Immunohistochemical staining was performed using the Autostainer universal staining system (DAKO-USA) (27). Five-μm formalin-fixed, paraffin-embedded sections from the tongues and neck lymph nodes of mice injected with different subpopulations of CA1 cells were stained with H&E or for p53 protein with a monoclonal specific antibody (DO-7 clone, titration 1:50; DAKO-Denmark).

Statistical analysis

All data are based on at least three experimental repeats unless otherwise stated, and are reported as mean ±SEM.

Results

A CD44<sup>high</sup>ESA<sup>low</sup> subpopulation exists in OSCC.

Flow cytometric analysis of the CA1 cell line demonstrated that a combination of staining for CD44 and ESA (epithelial-specific antigen, also known as EpCAM) consistently identified a CD44<sup>high</sup>ESA<sup>low</sup> subpopulation (Figure 1A). The co-expression of high CD44, typical of a CSC,
together with low expression of the epithelial marker, ESA, suggested the possible correspondence of
this population to an EMT CSC subpopulation. Cells with CD44^{high}\cdot ESA^{high}, CD44^{high}\cdot ESA^{low}, and
CD44^{low} expression patterns were therefore examined to establish their relationships respectively to a)
holoclone-forming, non-EMT CSCs, b) CSCs that have undergone an EMT, and c) paraclone-forming
cells that lack the capacity to self-renew.

**Morphological and behavioural differences between cell subpopulations in adherent and
suspension cultures point to two distinct CSC phenotypes.**

As previously described, OSCC cells (including the CA1 cell line) plated at low density in adherent
cultures form a range of colony morphologies, including self-renewing CD44^{high}\cdot ESA^{high} holoclone
and differentiating CD44^{low} paraclone colonies (23, 28). However, when cultures were examined in
more detail, individual elongated cells lying outside the compact holoclone colonies could be
identified (Figure S1A) and were found to have a CD44^{high}\cdot ESA^{low} staining pattern (Figure S1B).
When cell subpopulations were isolated by FACS and re-plated in culture, the CD44^{high}\cdot ESA^{low} cells
displayed an elongated fibroblast-like appearance and dispersal as individual cells, characteristics of
cells having undergone EMT (Figure 1B, middle). CD44^{high}\cdot ESA^{high} cells grew as holoclones (Figure
1B, left) and the CD44^{low} cells formed small abortive paraclone colonies (Figure 1B, right). Counting
the number of holoclones formed after plating the two non-EMT populations at clonal density
confirmed that the CD44^{high}\cdot ESA^{high} cells represent the holoclone-forming CSC type (Figure 1C).

Sorted populations of CD44^{high}\cdot ESA^{low} cells grew slower in adherent culture conditions than the
CD44^{high}\cdot ESA^{high} cells (Figure 1D). Time-lapse video demonstrated the high motility of these cells
(Movies S1A and S1B) and also suggested the generation and escape of motile cells at the periphery
of holoclone colonies. CD44<sup>high</sup>ESA<sup>low</sup> cells showed a much higher rate of migration in 3-dimensional in vitro Transwell migration assays (Figure 1E and S1C).

As ability to grow as floating spheres is a characteristic of CSCs in breast (29) and brain tumours (30), we counted the number of spheres formed when the three populations were seeded in suspension culture using non-adherent plates (Figure 1F). The CD44<sup>high</sup>ESA<sup>low</sup> cells formed 10 times more spheres than the CD44<sup>high</sup>ESA<sup>high</sup> cells, and 80 times more than the CD44<sup>low</sup> cells. The CD44<sup>high</sup>ESA<sup>low</sup> cells therefore represent a sub-population of CSCs greatly enriched for sphere forming ability. Laser capture of central and peripheral cell areas of epithelial colonies, followed by their transfer to suspension culture, indicated that the cells around colony peripheries form spheres (Figure S1D), whereas those in the centre of colonies yield no spheres, a further confirmation of the presence of CD44<sup>high</sup>ESA<sup>low</sup> EMT CSCs at the colony peripheries.

**An EMT-related gene expression pattern in the CD44<sup>high</sup>ESA<sup>low</sup> cells.**

To investigate the expression of EMT-related genes, we undertook quantitative RT-PCR of the CD44<sup>high</sup>ESA<sup>low</sup>, CD44<sup>high</sup>ESA<sup>high</sup> and CD44<sup>low</sup> populations. This showed (Figure 1G) that the CD44<sup>high</sup>ESA<sup>low</sup> cells have greater expression of Vimentin, Twist, Snail and Axl, all markers of EMT, and lower expression of the epithelial-specific genes E-cadherin, Calgranulin B, Involucrin and Keratin 15. Expression of Integrins α5 and β1 was moderately greater and expression of Integrin α6, C-myc and the Erbb2 and Erbb3 receptors was lower. Far smaller differences were seen between the CD44<sup>high</sup>ESA<sup>high</sup> and CD44<sup>low</sup> populations although CD44<sup>high</sup>ESA<sup>high</sup> cells showed moderately greater expression of Axl, and lower expression of Calgranulin B and Involucrin indicating less expression of epithelial differentiation markers. Antibody staining for Vimentin (Figure S1E) showed that the elongated cells around colony peripheries express Vimentin, whereas those inside the colonies do not.
TGF-β is a known inducer of EMT and, for further confirmation that the CD44\textsuperscript{high}ESA\textsuperscript{low} population represents cells having undergone EMT, we added TGF-β to cell cultures and examined the CD44 and ESA cell surface staining pattern (Figure 1H). After TGF-β addition the CD44\textsuperscript{high}ESA\textsuperscript{low} population approximately tripled in size, indicating that enhanced induction of EMT causes cells to acquire the CD44\textsuperscript{high}ESA\textsuperscript{low} staining pattern. Addition of SB431542, a potent and selective TGF-β inhibitor, completely reversed the ability of TGF-β to drive an enlargement of the CD44\textsuperscript{high}ESA\textsuperscript{low} population. Interestingly, however, SB431542 did not cause the size of the CD44\textsuperscript{high}ESA\textsuperscript{low} population to drop below that seen in the control cells, nor did it reverse the TGF-β induced EMT once established, indicating that TGF-β is not required for the maintenance of the EMT phenotype.

**EMT CSCs are present in cell lines generated from progressive stages of cutaneous SCC.**

We next investigated whether the CD44\textsuperscript{high}ESA\textsuperscript{low} EMT CSC population identified in OSCC is also present in cell lines derived from cutaneous SCC. Such cells were present in 3 matched specimens derived from the same patient (26) and measurement of the size of the CD44\textsuperscript{high}ESA\textsuperscript{low} populations in each of these three cell lines revealed stark differences between different stages of malignancy (Figure 2A). PM1, a cell line derived from pre-malignant dysplastic skin, did not contain a detectable CD44\textsuperscript{high}ESA\textsuperscript{low} population. Met1, a line derived from a primary SCC, contained a CD44\textsuperscript{high}ESA\textsuperscript{low} population representing 2.2±0.25% of the total population (Figure 2B). Met2, a line derived from a recurrent SCC arising at the same site, contained a CD44\textsuperscript{high}ESA\textsuperscript{low} population representing 29.7±5.73% of the total (Figure 2C).

Quantitative RT-PCR of FACS sorted populations from Met1 and Met2 (Figure 2D) showed patterns similar to those for OSCC. In both cell lines, the CD44\textsuperscript{high}ESA\textsuperscript{low} cells show greater expression of
Vimentin than the CD44<sup>high</sup>ESA<sup>high</sup> and ESA<sup>low</sup> cells, and have lower expression of E-cadherin, Calgranulin B, Involucrin, Keratin 15, Integrin α6, C-myc, Erbb2 and Erbb3. In Met2, the CD44<sup>high</sup>ESA<sup>low</sup> cells also have greater expression for Twist, Snail and Axl, perhaps suggesting that they have undergone a more complete EMT than the corresponding population in Met1. As with the CA1 OSCC line, only small differences were seen between the CD44<sup>high</sup>ESA<sup>high</sup> and CD44<sup>low</sup> populations in Met1 and Met2 (Figure 2D, bottom).

Adherent cultures of CD44<sup>high</sup>ESA<sup>low</sup> cells of both cell lines showed an elongated, fibroblast-like appearance (Figure 2E, middle), whereas CD44<sup>high</sup>ESA<sup>high</sup> cells grew as holoclones (Figure 2E, left), and CD44<sup>low</sup> cells formed small paraclone colonies (Figure 2E, right). As with OSCC, holoclone counts confirmed that the CD44<sup>high</sup>ESA<sup>high</sup> population represents the non-EMT CSC type (Figure 2F) and sphere formation in suspension culture indicated that in both lines the CD44<sup>high</sup>ESA<sup>low</sup> cells represent a subpopulation of CSCs greatly enriched for sphere forming ability (Figure 2G). In comparison to CD44<sup>high</sup>ESA<sup>high</sup> cells, CD44<sup>high</sup>ESA<sup>low</sup> cells proliferated at a slower rate in adherent culture (Figure 2H) and had a greater ability to migrate in a 3D <i>in vitro</i> Transwell migration assay (Figure 2I and S2), with the latter ability considerably more pronounced in Met2. The FACS sorted CD44<sup>high</sup>ESA<sup>high</sup> and CD44<sup>high</sup>ESA<sup>low</sup> cells could be passaged indefinitely, further indicating their ability to self-renew.

**EMT CSCs are present in cell populations freshly isolated from OSCC tumours.**

To determine whether an EMT CSC population exists in OSCC tumours <i>in vivo</i>, populations of cells were generated as explants from fresh samples of OSCC. After removal of fibroblasts, cells were FACS sorted on the basis of expression of CD44 and ESA, and were analysed by RT-PCR. Cells explanted from each of three tumours were found to contain a CD44<sup>high</sup>ESA<sup>low</sup> population that
represented 28.0%, 8.2% and 5.1% of the total population (Figure 3A-C). The CD44\textsuperscript{high}ESA\textsuperscript{low} cells of all three tumours showed greater expression of Vimentin and Twist and less expression of E-cadherin (Figure 3D).

The two CSC phenotypes exhibit distinct in vivo behaviours that reflect their in vitro properties.

Upon orthotopic injection into NOD/SCID mice, both CSC phenotypes show tumour initiating ability (Figure 4A). However, only the EMT CSCs exhibited any lymph node infiltration after 26 days (Figure 4A and S3A). Conversely, the non-EMT CSCs exhibited faster tumour growth at early time points (Figure 4B). Examination of tumours produced by both CSC phenotypes after 26 days by FACS (Figure S3B) and H&E staining (Figure S3C) demonstrated a return to a heterogenous cell population and no apparent difference in tumour histology, indicating that each CSC phenotype can re-populate the other in vivo.

There is switching between the two CSC phenotypes, and single cell cloning identifies a hierarchy of bipotent and unipotent EMT cells.

CD44\textsuperscript{high}ESA\textsuperscript{high} and CD44\textsuperscript{high}ESA\textsuperscript{low} populations from CA1 and Met1 were FACS sorted and single cell cloned to determine whether such clones give rise to both EMT and non-EMT cells, thus indicating bipotency of the clonal cell of origin. Clones were grown up, stained for CD44 and ESA, and analysed. The results (Figure 5) indicated that 100% of CD44\textsuperscript{high}ESA\textsuperscript{high} clones were bipotent as indicated by the production of both non-EMT and EMT cell populations. Conversely, not all CD44\textsuperscript{high}ESA\textsuperscript{low} clones were bipotent; only a fraction of the CD44\textsuperscript{high}ESA\textsuperscript{low} clones (50% in CA1 and
29% in Met1) produced mixed populations. The rest were unipotent and, despite several subsequent rounds of passaging, gave rise only to EMT cells.

**CD44^{high}ESA^{low/+} marker expression identifies the bipotent EMT CSCs.**

We investigated whether differences in levels of ESA expression might distinguish bipotent from unipotent EMT cells present in SCC. We FACS sorted CD44^{high}ESA^{low/+} and CD44^{high}ESA^{low/-} cell populations from CA1 for single cell cloning (illustrated in Figure 6A). Clones were grown up and assessed by FACS for the presence of both non-EMT and EMT cell populations. The results indicated that none of the CD44^{high}ESA^{low/-} clones was bipotent but that heterogeneity was regenerated by 44% of the CD44^{high}ESA^{low/+} clones (Figure 6B), showing that the EMT CSCs capable of undergoing MET lie at the ESA^{high} end of the CD44^{high}ESA^{low} population. Analysis of sphere forming abilities for each of the CA1, Met1 and Met2 cell lines indicated similar numbers of spheres produced by CD44^{high}ESA^{low/+} and CD44^{high}ESA^{low/-} populations (Figure 6C). Therefore, despite differences in plasticity these populations have similar abilities to grow as floating spheres. A single cell clone created from a CA1 bipotent EMT CSC was FACS sorted for single cell cloning of the CD44^{high}ESA^{low/+} and CD44^{high}ESA^{low/-} cell populations (Figure S4A). 92% of the CD44^{high}ESA^{low/+} clones were bipotent whereas only 17% of the CD44^{high}ESA^{low/-} clones were bipotent. The presence of a proportion of clones that were unipotent demonstrates that the cloned bipotent EMT CSC gave rise directly to unipotent EMT cells.

**The CD44^{high}ESA^{low/+} bipotent EMT CSCs are ALDH1+.**

Interestingly, we found that CD44^{high}ESA^{low/+} cells show considerably higher activity than CD44^{high}ESA^{low/-} cells for the CSC marker ALDH1 (Figure 6D). We therefore investigated a possible
link between the bipotency of EMT CSCs and their ALDH1 activity. For each cell line, CD44\textsuperscript{high}ESA\textsuperscript{low} cells were fractionated on the basis of ALDH1 activity (Figure 6E) and then assayed for the number of non-EMT cells they produce in culture. The results (Figure 6F) show that after growing up from clonal density the percentage of non-EMT cells was consistently greater for the CD44\textsuperscript{high}ESA\textsuperscript{low} ALDH1\textsuperscript{+} cells than for the CD44\textsuperscript{high}ESA\textsuperscript{low} ALDH1\textsuperscript{−} cells; 6.9 times, 27.4 times and 5.9 times greater in CA1, Met1 and Met2 respectively. Therefore, high ALDH1 expression marks bipotent EMT CSCs. As for CD44\textsuperscript{high}ESA\textsuperscript{low/+} and CD44\textsuperscript{high}ESA\textsuperscript{low/-} cells, both bipotent CD44\textsuperscript{high}ESA\textsuperscript{low} ALDH1\textsuperscript{+} cells and unipotent CD44\textsuperscript{high}ESA\textsuperscript{low} ALDH1\textsuperscript{−} cells formed similar numbers of spheres in suspension culture (Figure 6G). Suspension culture did not enable the unipotent CD44\textsuperscript{high}ESA\textsuperscript{low} ALDH1\textsuperscript{−} cells to change their phenotype (Figure S4B), indicating that their unipotent state is not an artefact of adherent culture. Assessment of four bipotent and four unipotent single cell clones formed from CD44\textsuperscript{high}ESA\textsuperscript{low} Met1 cells indicated that the CD44\textsuperscript{high}ESA\textsuperscript{low} cells in the bipotent clones had consistently greater ALDH1 activity than those in the unipotent clones (Figure S4C), further confirming a link between bipotent EMT CSCs and ALDH1 positivity.

**Discussion**

Our investigations demonstrate that cultures of malignant cell populations consistently contain motile cells with a fibroblast-like morphology and that such cells stain strongly for CD44 but only weakly for ESA. Cells with a CD44\textsuperscript{high}ESA\textsuperscript{low} phenotype isolated from OSCC cell lines, from fresh samples of OSCC tumour, and from primary and recurrent cutaneous SCC, express Vimentin, Twist and Axl, genes that act as markers of EMT, and show reduced expression of epithelial markers such as e-cadherin, Involucrin and CK15. These cells proliferate slowly, are more migratory, and when grown in suspension culture they display the sphere forming ability that has previously been associated with stem cells (31). We also demonstrate a separate CD44\textsuperscript{high}ESA\textsuperscript{high} cell population that, when isolated
by FACS, is highly proliferative and forms holoclone colonies in adherent culture that are typical of epithelial stem cells. Injection into NOD/SCID indicates that the attributes demonstrated by these cells in vitro are maintained in vivo. Taken together, these findings indicate that SCCs contain CSCs with two distinct phenotypes, one similar to normal epithelial stem cells, and another similar to the EMT CSCs described by Mani et al. (16). These findings fit well with the “migrating cancer stem cell” concept of Brabletz (32) which requires malignant stem cells to acquire two phenotypes; one that is associated with growth and another that is migratory and characterized by “transient expression of epithelial to mesenchymal transition-associated genes, which can be reversed by a mesenchymal to epithelial transition (MET), leading to epithelial redifferentiation”, and thus enables secondary tumour formation at a metastatic site.

We propose a malignant stem model that defines the hierarchical cellular relationships within the tumour. In this model (Figure 7), CD44^{high}ESA^{high} holoclone-forming non-EMT CSCs have division patterns directed largely to self-renewal but they also generate cells entering two distinct pathways of differentiation. One type loses CD44 expression, lacks self-renewal ability and forms paraclones, a change interpreted as entry into an abortive epithelial terminal differentiation pathway. The other cell type is CD44^{high}ESA^{low}, is migratory, and has expression patterns and behaviour indicative of EMT. FACS isolation of populations of non-EMT and EMT cells indicates that the cells of one population regenerate cells of the other through reciprocal processes of EMT and MET, and this phenotypic plasticity is maintained in vivo; therefore, it seems that CSCs have the ability to take on an EMT phenotype for migration to a secondary site, before reverting back to the proliferative non-EMT phenotype to enable formation of a metastatic tumour at that secondary site. Single cell cloning indicates that ability to undergo MET is restricted to a sub-population of the EMT cells that is marked by the CD44^{high}ESA^{low/+/+ALDH^+} expression pattern. Thus, while the non-EMT population demonstrates a differentiation hierarchy that can be assayed as loss of self-renewal capacity, the EMT
population has a secondary differentiation hierarchy that can be assayed in terms of the plasticity required to reconstitute tumour heterogeneity. As migrating EMT cells undergo an MET in order to establish a new epithelial metastasis, it seems that it is the CD44\textsuperscript{high}ESA\textsuperscript{low/+}ALDH\textsuperscript{+} EMT cells that are endowed with the greatest metastatic potency. These results may explain the observation that metastatic ability of breast cancer cells is restricted to ALDH1\textsuperscript{+} cells (1).

Analyses of the properties of CSCs are largely dependent on the ability to accurately identify and assay them. We show that clonogenic assays under adherent conditions report the content of CD44\textsuperscript{high}ESA\textsuperscript{high} cells but that tumour sphere formation represents the CD44\textsuperscript{high}ESA\textsuperscript{low} population. As CD44\textsuperscript{high}ESA\textsuperscript{low} cells usually represent only a minor fraction of the total CSC population, sphere forming assays will significantly underestimate the total number of self-renewing CSCs present. Conversely, assays of clonogenicity may exclude the EMT population. These results highlight the importance of using multiple methods for assaying the CSC content within a heterogenous tumour cell population.

The demonstration that EMT and non-EMT CSCs co-exist in pathological tissues identifies potentially important new targets for therapeutic interventions intended to halt tumour recurrence and metastatic spread. EMT cells show resistance to conventional chemotherapy in breast cancer (4, 19) and SCC (18), but drug screening has disclosed the existence of agents that are selectively toxic to EMT cells and suggests that such resistance can be overcome (4). Differential properties of EMT and non-EMT cells, such as downregulation of Erbb2 and Erbb3 in EMT cells, suggests they may also vary in their resistance to inhibitors of EGFR and other receptor families. Direct analyses of the responses of EMT and non-EMT CSCs are required and the ability to assess these in vitro may enable more rapid development of combinational therapies that act effectively on the entire CSC population.
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Figure Legends

Figure 1. The CA1 OSCC cell line contains cancer stem cells that have undergone EMT.
(A) FACS sorting of CA1 cells by expression of CD44 and ESA. Sorted populations are gated.
(B) Phase-contrast images of the populations shown in (A), after being plated at clonal density (1000cells/ml) and allowed to grow for 7 days.
(C) Holoclone counts for the CD44^{high}ESA^{high} and CD44^{low} populations, after plating at clonal density.
(D) Cell counts (cells/ml) for the CD44^{high}ESA^{high} and CD44^{high}ESA^{low} populations, 10 days after plating at clonal density.
(E) The number of migrated cells from the CD44^{high}ESA^{high} and CD44^{high}ESA^{low} populations in Transwell migration assays.
(F) Left; sphere counts for the populations shown in (A). Right; a typical CA1 sphere.
(G) QPCR analysis of the populations shown in (A). Top; gene expression in CD44^{high}ESA^{low} cells relative to that in CD44^{low} cells. Bottom; gene expression in CD44^{high}ESA^{high} cells relative to that in CD44^{low} cells.
(H) The size of the CD44^{high}ESA^{low} population (as % of total cell number) after 5 days of treatment with TGF-β or the TGF-β inhibitor SB431542.

Figure 2. Cell lines from progressive stages of cutaneous SCC contain cancer stem cells that have undergone EMT.
(A) Graph showing the % of cells in the CD44^{high}ESA^{low} population of three cell lines representing progression of cutaneous SCC.
(B and C) FACS sorting of MET1 (B) and MET2 (C) cells by expression of CD44 and ESA. Sorted populations are gated and shown as % of total cells.

(D) QPCR analysis of the populations shown in (B) and (C). Top; gene expression in CD44\textsuperscript{high}ESA\textsuperscript{low} cells relative to that in CD44\textsuperscript{low} cells. Bottom; gene expression in CD44\textsuperscript{high}ESA\textsuperscript{high} cells relative to that in CD44\textsuperscript{low} cells.

(E) Phase-contrast images of the populations shown in (B) and (C), after being plated at clonal density (1000 cells/ml) and allowed to grow for 7 days.

(F) Holoclone counts for the CD44\textsuperscript{high}ESA\textsuperscript{high} and CD44\textsuperscript{low} populations, after plating at clonal density.

(G) Sphere counts for the populations shown in (B) and (C). Representative MET1 and MET2 spheres are shown.

(H) Cell counts (cells/ml) for the CD44\textsuperscript{high}ESA\textsuperscript{high} and CD44\textsuperscript{high}ESA\textsuperscript{low} populations, 10 days after plating at clonal density.

(I) The number of migrated cells from the CD44\textsuperscript{high}ESA\textsuperscript{high} and CD44\textsuperscript{high}ESA\textsuperscript{low} populations in Transwell migration assays.

**Figure 3. EMT CSCs in fresh OSCC tumours.**

(A, B and C) FACS sorting by expression of CD44 and ESA for cells generated from 3 OSCC tumours, termed (A) LUC4, (B) LUC9 and (C) LUC11. The CD44\textsuperscript{high}ESA\textsuperscript{low} population is gated and shown as % of total cells.

(D) QPCR analysis of the cells generated from the 3 tumours; gene expression in the gated CD44\textsuperscript{high}ESA\textsuperscript{low} population relative to that in the rest of the cells.

**Figure 4. EMT CSCs drive metastatic dissemination and non-EMT CSCs drive tumour growth in vivo.**
(A) Tumour incidence and rate of lymph node infiltration from orthotopic tongue injections of CA1 cells.

(B) Tumour growth rate after orthotopic tongue injection of 5000 CA1 cells. n=2.

**Figure 5. Single cell cloning of the CSC populations.**

(A) FACS sorting of CA1 (top) and MET1 (bottom) cells by expression of CD44 (y-axis) and ESA (x-axis), for single cell cloning of the gated populations. To the sides are representative FACS plots of populations produced by the single cell clones.

(B) Table showing the number of single cell clones from the two FACS sorted populations which were able to give rise to both non-EMT and EMT cells.

**Figure 6. CD44$^{\text{high}}$ESA$^{\text{low/+}}$ALDH1$^{+}$ marker expression identifies the EMT CSCs that are capable of reconstituting tumour heterogeneity.**

(A) FACS sorting of CA1 cells by expression of CD44 (y-axis) and ESA (x-axis), for single cell cloning of the gated CD44$^{\text{high}}$ESA$^{\text{low/+}}$ and CD44$^{\text{high}}$ESA$^{\text{low/-}}$ populations.

(B) Table showing the number of single cell clones from the two FACS sorted populations described in (A) which were able to give rise to both non-EMT and EMT cells.

(C) Sphere counts for the CD44$^{\text{high}}$ESA$^{\text{low/+}}$ and CD44$^{\text{high}}$ESA$^{\text{low/-}}$ populations from CA1, MET1 and MET2.

(D) The percentage of the CD44$^{\text{high}}$ESA$^{\text{low/+}}$ and CD44$^{\text{high}}$ESA$^{\text{low/-}}$ cells that are ALDH1$^{+}$ (using the method described in Fig. 6E) in the CA1, MET1 and MET2 lines.

(E) Representative FACS sort for the CA1 line, showing how the CD44$^{\text{high}}$ESA$^{\text{low}}$ population was selected and then fractionated on the basis of ALDH1 expression (ALDH1-FITC on the x-axis of the right-hand plots). The Aldeflour assay inhibitor DEAB was used to enable negative control staining.
for the setting of gates (top right), and then ALDH1$^+$ and ALDH1$^-$ cells were selected in the absence of the inhibitor (bottom right).

(F) The ability to reconstitute the heterogeneous population, as shown by the production of non-EMT cells 10 days after the FACS sorted populations described in (E) were plated at clonal density.

(G) Sphere counts for the populations described in (E).

**Figure 7. A model showing the different cell types present in SCC, their marker expression, and their relationships to one another.**
Figure 4

A

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Number of cells</th>
<th>Tumour incidence</th>
<th>Lymph node infiltration</th>
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<tr>
<td>CD14&lt;sup&gt;high&lt;/sup&gt;ESA&lt;sup&gt;high&lt;/sup&gt;</td>
<td>10000</td>
<td>4 out of 4</td>
<td>0 out of 4</td>
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<td></td>
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<td>2 out of 2</td>
<td>not tested</td>
</tr>
<tr>
<td></td>
<td>1000</td>
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<td>0 out of 4</td>
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<tr>
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<td>5000</td>
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<td>not tested</td>
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<tr>
<td></td>
<td>1000</td>
<td>1 out of 4</td>
<td>1 out of 4</td>
</tr>
</tbody>
</table>

B

![Graph showing tumor incidence over days after injection for different cell subpopulations.](image)
Figure 6

A

B

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Subpopulation</th>
<th>Number of clones that can restore heterogeneity (%)</th>
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<tr>
<td>CA1</td>
<td>CD44&lt;sup&gt;+/+&lt;/sup&gt;ESA&lt;sup&gt;dim&lt;/sup&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>8 out of 18 (44)</td>
</tr>
<tr>
<td></td>
<td>CD44&lt;sup&gt;-/-&lt;/sup&gt;ESA&lt;sup&gt;dim&lt;/sup&gt;</td>
<td>0 out of 19 (0)</td>
</tr>
</tbody>
</table>

C

D

E

F

G
Figure 7

Non-EMT phenotype

Bipotent
CD44+/ESA+/ALDH1-

Lack self-renewal
CD44-

EMT

MET

CD44+/ESA+/ALDH1-

Bipotent

Unipotent
CD44+/ESA+/ALDH1-

EMT phenotype
Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative


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