Cancer Stem Cells in Squamous Cell Carcinoma Switch between Two Distinct Phenotypes That Are Preferentially Migratory or Proliferative

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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 (CSC) 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 CD44highESAhigh, was proliferative and retained epithelial characteristics (non-EMT CSCs), whereas the other phenotype, termed CD44highESAlow, 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+ (ALDH1+), implying that only ALDH1+ 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. Cancer Res; 71(15); 5317–26. ©2011 AACR.

Introduction

Several studies have implicated cancer stem cells (CSC) in tumor invasion and metastasis (1, 2) and have related tumor recurrence after therapy to therapeutic resistance of CSCs (3, 4). Typically, CSCs are defined as a subpopulation of tumor cells having both tumor-initiating ability and the ability to reconstitute the cellular heterogeneity typical of the original tumor (5). For solid tumors, CSCs with these properties were first shown in breast cancers as cells with a CD44+CD24lowESA+ staining pattern (6). As for several other malignancies, a subpopulation of CD44+ cells with CSC properties has been identified within oral squamous cell carcinoma (OSCC; Ref. 7). In breast cancer and OSCC, subpopulations 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 TGF-β can induce EMT and lead to downregulation 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 tumor environment (15). Breast-cancer cells with the CD44+CD24lowCD133+ tumor-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 tumors (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 paraclines (22). Similar clonal patterns are present in cell lines generated from OSCC (23) and also from prostate cancers where holoclone cells, but not paracline cells, are capable of forming tumors in mouse xenograft models (24). Here we show 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 coexist 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 tumors. We also show 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 pre-malignant 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, 0.75 cm² tissue culture wells were coated with polyHEMA (Sigma) (12 mg/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 μmol/L SB431542 (Sigma) was added either simultaneously with the TGF-β additions or for 5 days following 5 days of TGF-β treatment.

Fluorescence-activated cell sorting and immunofluorescence
For fluorescence-activated cell sorting (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 μg/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 ALDH activity, cells were stained with Aldefluor reagent (Aldegen) 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 permeabilized with 0.1% Triton-X prior to addition of DAPI at 1 μg/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 (8 μm 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 tumor 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<sup>high</sup>ESA<sup>high</sup> and CD44<sup>high</sup>-ESA<sup>low</sup> 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<sup>high</sup>ESA<sup>low</sup>/<sup>+</sup> and CD44<sup>high</sup>-ESA<sup>low</sup>/<sup>–</sup> 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.

Tumorigenesis assays in mice
For these assays, which were performed at the Gade Institute in Bergen, nonobese diabetic/severe combined immunodeficiency (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 50 μL of Matrigel (BD Biosciences) and transplanted into the tongue. Immunohistochemical staining was carried out using the Autostainer universal staining system (DAKO-USA; Ref. 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 CD44highESAlow subpopulation exists in oral squamous cell carcinoma
Flow cytometric analysis of the CA1 cell line showed that a combination of staining for CD44 and ESA (epithelial-specific antigen, also known as EpCAM) consistently identified a CD44highESAlow subpopulation (Fig. 1A). The coexpression 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 CD44highESAhigh, CD44highESAlow, and CD44low 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 behavioral differences between cell subpopulations in adherent and suspension cultures point to two distinct cancer stem cell 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 CD44highESAhigh holoclone and differentiating CD44low paraclone colonies (23, 28). However, when cultures were examined in more detail, individual elongated cells lying outside the compact holoclone colonies could be identified (Supplementary Fig. S1A) and were found to have a CD44highESAlow staining pattern (Supplementary Fig. S1B). When cell subpopulations were isolated by FACS and replated in culture, the CD44highESAlow cells displayed an elongated fibroblast-like appearance and dispersal as individual cells, characteristics of cells having undergone EMT (Fig. 1B, middle). CD44highESAhigh cells grew as holoclones (Fig. 1B, left) and the CD44low cells formed small abortive paraclone colonies (Fig. 1B, right). Counting the number of holoclones formed after plating the two non-EMT populations at clonal density...
confirmed that the CD44<sup>high</sup>ESA<sup>low</sup> cells represent the holo-
clone-forming CSC type (Fig. 1C).

Sorted populations of CD44<sup>high</sup>ESA<sup>low</sup> cells grew slower in
adherent culture conditions than the CD44<sup>high</sup>ESA<sup>high</sup> cells (Fig. 1D). Time-lapse video showed the high motility of these
cells (Supplementary 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 (Fig. 1E and Supplementary Fig. S1C).

As ability to grow as floating spheres is a characteristic of
CSCs in breast (29) and brain tumors (30), we counted the
number of spheres formed when the three populations were
seeded in suspension culture using nonadherent plates
(Fig. 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 subpopulation 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 (Supplementary Fig. S1D), whereas
those in the center 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 epithelial-to-mesenchymal transition–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
(Fig. 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 (Supplementary
Fig. S1E) showed that the elongated cells around colony
peripheries express Vimentin, whereas those inside the colo-
nies do not.

TGF-β is a known inducer of EMT and, for further con-
firmation that the CD44<sup>high</sup>ESA<sup>low</sup> population represents cells
having undergone EMT, we added TGF-β to cell cultures and
examined the CD44 and ESA cell surface-staining pattern
(Fig. 1H). After TGF-β addition the CD44<sup>high</sup>ESA<sup>low</sup> population
approximately tripled in size, indicating that enhanced induc-
tion of EMT causes cells to acquire the CD44<sup>high</sup>ESA<sup>low</sup>
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<sup>high</sup>ESA<sup>low</sup> population. Interest-
ingly, however, SB431542 did not cause the size of the
CD44<sup>high</sup>ESA<sup>low</sup> population to drop below that seen in the
cell cultures, 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.

**Epithelial-to-mesenchymal transition cancer stem cells
are present in cell populations generated from progressive
stages of cutaneous squamous cell carcinoma**

We next investigated whether the CD44<sup>high</sup>ESA<sup>low</sup> 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<sup>high</sup>ESA<sup>low</sup> populations in
each of these three cell lines revealed stark differences
between different stages of malignancy (Fig. 2A). PM1, a cell
line derived from premalignant dysplastic skin, did not con-
tain a detectable CD44<sup>high</sup>ESA<sup>low</sup> population. Met1, a line
derived from a primary SCC, contained a CD44<sup>high</sup>ESA<sup>low</sup>
population representing 2.2 ± 0.25% of the total population
(Fig. 2B). Met2, a line derived from a recurrent SCC arising
at the same site, contained a CD44<sup>high</sup>ESA<sup>low</sup> population
representing 29.7 ± 5.73% of the total (Fig. 2C).

Quantitative RT-PCR of FACS sorted populations from
Met1 and Met2 (Fig. 2D) showed patterns similar to those for
OSCC. In both cell lines, the CD44<sup>high</sup>ESA<sup>low</sup> cells show
greater expression of Vimentin than the CD44<sup>high</sup>ESA<sup>high</sup>
and CD44<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 corre-
sponding 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 (Fig. 2D, bottom).

Adherent cultures of CD44<sup>high</sup>ESA<sup>low</sup> cells of both cell lines
showed an elongated, fibroblast-like appearance (Fig. 2E,
middle), whereas CD44<sup>high</sup>ESA<sup>high</sup> cells grew as holoclones
(Fig. 2E, left), and CD44<sup>low</sup> cells formed small paraclone
colonies (Fig. 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 (Fig. 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 activity (Fig. 2G). In com-
parison to CD44<sup>high</sup>ESA<sup>high</sup> cells, CD44<sup>high</sup>ESA<sup>low</sup> cells pro-
liferated at a slower rate in adherent culture (Fig. 2H) and
had a greater ability to migrate in a 3D in vitro Transwell
migration assay (Fig. 2I and Supplementary Fig. 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.

**Epithelial-to-mesenchymal transition cancer stem cells
are present in cell populations freshly isolated from
oral squamous cell carcinoma tumors**

To determine whether an EMT CSC population exists in
OSCC tumors in vivo, 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 analyzed by RT-PCR. Cells explanted from each of three tumors were found to contain a CD44 high ESA low population that represented 28.0%, 8.2%, and 5.1% of the total population (Fig. 3A–C). The CD44 high ESA low cells of all three tumors showed greater expression of Vimentin and Twist and less expression of E-cadherin (Fig. 3D).

The two cancer stem cell phenotypes exhibit distinct in vivo behaviors that reflect their in vitro properties

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

There is switching between the two cancer stem cells phenotypes, and single-cell cloning identifies a hierarchy of bipotent and unipotent epithelial-to-mesenchymal transition cells

CD44 high ESA high and CD44 high ESA 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 analyzed. The results (Fig. 5) indicated that 100% of CD44 high ESA high clones were bipotent as indicated by the production of both non-EMT and EMT cell populations. Conversely, not all CD44 high ESA low clones were bipotent; only a fraction of the CD44 high ESA 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.
CD44highESAlo/þ marker expression identifies the bipotent epithelial-to-mesenchymal transition cancer stem cells

We investigated whether differences in levels of ESA expression might distinguish bipotent from unipotent EMT cells present in SCC. We FACS sorted CD44highESAlo/þ and CD44highESAlo/C0 cell populations from CA1 for single-cell cloning (illustrated in Fig. 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 CD44highESAlo/C0 clones were bipotent but that heterogeneity was regenerated by 44% of the CD44highESAlo/þ clones (Fig. 6B), showing that the EMT CSCs capable of undergoing MET lie at the ESAhigh end of the CD44highESAlo population.

Analysis of sphere forming abilities for each of the CA1, Met1, and Met2 cell lines indicated similar numbers of spheres produced by CD44highESAlo/þ and CD44highESAlo/C0 populations (Fig. 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 CD44highESAlo/þ and CD44highESAlo/C0 cell populations (Supplementary Fig. S4A). 92% of the CD44highESAlo/þ clones were bipotent whereas only 17% of the CD44highESAlo/C0 clones were bipotent. The presence of a proportion of clones that were unipotent shows that the cloned bipotent EMT CSC gave rise directly to unipotent EMT cells.

The CD44highESAlo/þ bipotent epithelial-to-mesenchymal transition cancer stem cells are ALDH1þ

Interestingly, we found that CD44highESAlo/þ cells show considerably higher activity than CD44highESAlo/C0 cells for the CSC marker ALDH1 (Fig. 6D). We therefore investigated a possible link between the bipotency of EMT CSCs and their ALDH1 activity. For each cell line, CD44highESAlo cells were fractionated on the basis of ALDH1 activity (Fig. 6E) and then assayed for the number of non-EMT cells they produce in culture. The results

Figure 3. EMT CSCs in fresh OSCC tumors. A, B, and C, FACS sorting by expression of CD44 and ESA for cells generated from 3 OSCC tumors, termed (A) LUC4, (B) LUC9, and (C) LUC11. The CD44highESAlo/þ population is gated and shown as % of total cells. D, QPCR analysis of the cells generated from the 3 tumors; gene expression in the gated CD44highESAlo/þ population relative to that in the rest of the cells.

Figure 4. EMT CSCs drive metastatic dissemination and non-EMT CSCs drive tumor growth in vivo. A, tumor incidence and rate of lymph node infiltration from orthotopic tongue injections of CA1 cells. B, tumor growth rate after orthotopic tongue injection of 5000 CA1 cells. n = 2.
(Fig. 6F) show that after growing up from clonal density the percentage of non-EMT cells was consistently greater for the CD44<sup>high</sup>ESA<sup>low</sup>ALDH<sup>+</sup> cells than for the CD44<sup>high</sup>ESA<sup>low</sup>ALDH<sup>-</sup> 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<sup>high</sup>ESA<sup>low</sup> and CD44<sup>high</sup>ESA<sup>low</sup>/C<sup>0</sup> cells, both bipotent CD44<sup>high</sup>ESA<sup>low</sup>ALDH<sup>+</sup> cells and unipotent CD44<sup>high</sup>ESA<sup>low</sup>ALDH<sup>-</sup> cells formed similar numbers of spheres in suspension culture (Fig. 6G). Suspension culture did not enable the unipotent CD44<sup>high</sup>ESA<sup>low</sup>ALDH<sup>-</sup> cells to change their phenotype (Supplementary Fig. S4B), indicating that their unipotent state is not an artefact of adherent culture. Assessment of 4 bipotent and four unipotent single cell clones formed from CD44<sup>high</sup>ESA<sup>low</sup>Met1 cells indicated that the CD44<sup>high</sup>ESA<sup>low</sup> cells in the bipotent clones had consistently greater ALDH1 activity than those in the unipotent clones (Supplementary Fig. S4C), further confirming a link between bipotent EMT CSCs and ALDH1 positivity.

Discussion

Our investigations show 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<sup>high</sup>ESA<sup>low</sup> phenotype isolated from OSCC cell lines, from fresh samples of OSCC tumor, 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 show a separate CD44<sup>high</sup>ESA<sup>high</sup> cell population that, when isolated by FACS, is highly proliferative and forms holoclone colonies in adherent culture that are indefinitely self-renewing and typical of epithelial stem cells. Injection into NOD/SCID mice indicates that the attributes showed 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 and colleagues (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 tumor formation at a metastatic site.

We propose a malignant stem cell model that defines the hierarchical cellular relationships within the tumor. In this...
model (Fig. 7). CD44<sup>high</sup>ESA<sup>high</sup> 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<sup>high</sup>ESA<sup>low</sup>, is migratory, and has expression patterns and behavior 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 tumor at that secondary site. Single-cell cloning indicates that ability to undergo MET is restricted to a subpopulation of the EMT cells that is marked by the CD44<sup>high</sup>ESA<sup>low</sup>/ALDH<sup>high</sup> expression pattern. Thus, whereas the non-EMT population shows a differentiation pattern 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 tumor heterogeneity. As migrating EMT cells undergo an MET to establish a new epithelial metastasis, it seems that it is the CD44<sup>high</sup>ESA<sup>low</sup>/ALDH<sup>low</sup> 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 ALDH<sup>1+</sup> 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<sup>high</sup>ESA<sup>high</sup> cells but that tumor sphere formation represents the CD44<sup>high</sup>ESA<sup>low</sup> population. As CD44<sup>high</sup>ESA<sup>low</sup> 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 heterogeneous tumor-cell population.

The demonstration that EMT and non-EMT CSCs co-exist in pathologic tissues identifies potentially important new targets for therapeutic interventions intended to halt tumor 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 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.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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