Objective: To design in vitro and in vivo models of metastasis to study the behavior of cancer stem cells (CSCs) in head and neck squamous cell carcinoma (HNSCC).

Design: Cells were sorted for CD44 expression using flow cytometry. Sorted cells were used in an in vitro invasion assay. For in vivo studies, CSCs and non-CSCs were injected into the tail veins of mice, and lungs were either harvested or imaged to evaluate for lesions.

Results: In vitro, CD44<sup>high</sup> cells were more motile but not more invasive than CD44<sup>low</sup> cells. In vivo, 8 of 17 mice injected with CD44<sup>high</sup> cells and 0 of 17 mice injected with CD44<sup>low</sup> cells developed lung lesions. Two of the lesions arose from CSCs from a primary tumor and 6 from CSCs from HNSCC cell lines.

Conclusions: In vitro, CSCs do not have an increased ability to invade through basement membrane, but they migrate more efficiently through a porous barrier. In contrast, CSCs efficiently formed lung lesions in vivo, whereas non-CSCs did not give rise to any distant disease. This phenomenon could be due to the enhanced migratory capacity of CSCs, which may be more important than basement membrane degradation in vivo.
disseminated tumor cells (DTCs) in their bone marrow. With the use of CD44+/CD24− co-staining, the majority of the DTCs were found to express a CSC phenotype. A link between metastasis and stemlike cells has also been shown in pancreatic cancer, in which co-staining with the CSC marker CD133 and CXCR4 has been used to identify a metastatic phenotype. To help elucidate the role of head and neck CSCs in the spread of malignant cells outside the primary tumor bed, we designed both in vitro and in vivo models of metastasis to study the behavior of this unique tumor cell subpopulation.

METHODS

After obtaining informed consent, tumors were obtained from subjects at the University of Michigan hospital, Ann Arbor. Animal care and experimental protocols were performed in accordance with procedures and guidelines established by the University Committee on the Use and Care of Animals and the Unit for Laboratory Animal Medicine.

CELL CULTURE

The following HNSCC cells lines were used: UMSCC-6, a base of tongue tumor from a male patient; UMSCC-10A, a tumor of the true vocal cord from a male patient; UMSCC-12, a laryngeal cancer from a male patient; UMSCC-14A, a floor of the mouth tumor from a female patient; UMSCC-14B, a recurrence of the same cancer; UMSCC-47, a lateral tongue cancer from a male patient; HN-111, a primary tumor of the lateral tongue from a female patient; and UMSCC-103, the cell line derived from HN-111 (Table 1). Cells were grown in Dulbecco Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% nonessential amino acids.

LUCIFERASE TRANSDUCTION

The cell lines UMSCC-47, UMSCC-12, and UMSCC-14B were transduced with human immunodeficiency virus (HIV) with a luciferase reporter, a lentiviral vector containing a pLentilox concentration of 2.5 × 105 cells/mL (for primary tumor cells). A total volume of up to 200 uL of cell suspension was injected into the tail veins of nonobese diabetic severe combined immunodeficient (NOD-SCID) mice. After the injections with UMSCC-6 and HN-111, CD44high and CD44low cells from HN-111, UMSCC-6, UMSCC-47-Luc, UMSCC-12-Luc, and UMSCC-14B-Luc were resuspended in phosphate-buffered saline to a concentration of 2.5 × 105 cells/mL (for cell lines) or 5.0 × 104 cells/mL (for primary tumor cells). A total volume of up to 200 uL of cell suspension was injected into the tail veins of nonobese diabetic severe combined immunodeficient (NOD-SCID) mice. After the injections with UMSCC-6 and HN-111, mice were humanely killed at 6 months to evaluate for the presence of metastases. Lungs were harvested, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. A pathologist confirmed the presence or absence of metastases (Table 2).

BIOOLUMINESCENT IMAGING

Two months after tail vein injections with UMSCC-47-Luc, UMSCC-12-Luc, and UMSCC-14B-Luc, mice were taken for bioluminescent imaging. Luciferin (100 uL of a 40-mg/mL suspension) was injected intraperitoneally into each mouse approximately 10 minutes prior to imaging. Mice were anesthetized with isoflurane and imaged in a Xenogen IVIS 200 (Caliper Life Sciences, Hopkinton, Massachusetts) (Figure 3).

BOYDEN MIGRATION CHAMBERS

Two hours prior to use, Matrigel-coated invasion chambers were rehydrated by incubation with DMEM at 37°C. CD44high and CD44low HICS. The suspensions were then incubated with either anti-CD44 antibody (allophycocyanin-conjugated, mouse antihuman, clone G44-26; BD Pharmingen, San Diego, California) or mouse IgG2b, κ isotype control antibody (allophycocyanin-conjugated, clone 27-33; BD Pharmingen), both used at a 1:50 dilution for 15 to 20 minutes, or no antibody. For the primary tumor, lineage markers anti-CD2, -CD3, -CD10, -CD16, -CD18, -CD31, -CD45, and -CD140b (all diluted 1:50; BD Pharmingen) were used to allow identification of contaminating non-tumor cells. Cells were then washed and resuspended in HBSS–2% HICS containing the dead cell counterstain 4',6-diamidino-2-phenylindole (DAPI; BD Pharmingen) at 105 cells per 0.5 mL, then immediately placed on ice for analysis and sorting. Gates for fluorescence-activated cell sorting were set as follows: the cells incubated without antibody were used to account for autofluorescence, and the cells incubated with the isotype control antibody were used to control for nonspecific binding (Figure 1 C and D). The 10% to 15% of cells with the highest and lowest CD44 expression were collected for use (Figure 2).

Table 1. Characteristics of HNSCC Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Patient Gender</th>
<th>Specimen Site</th>
<th>TNM Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMSCC-6</td>
<td>M</td>
<td>BOT</td>
<td>T2N0M0</td>
</tr>
<tr>
<td>UMSCC-10A</td>
<td>M</td>
<td>True vocal cord</td>
<td>T3N0M0</td>
</tr>
<tr>
<td>UMSCC-12</td>
<td>M</td>
<td>Larynx</td>
<td>T2N1M0</td>
</tr>
<tr>
<td>UMSCC-14A</td>
<td>F</td>
<td>FOM</td>
<td>T1N0M0</td>
</tr>
<tr>
<td>UMSCC-14B</td>
<td>F</td>
<td>FOM</td>
<td>T1N0M0</td>
</tr>
<tr>
<td>UMSCC-47</td>
<td>M</td>
<td>Lateral tongue</td>
<td>T3N1M0</td>
</tr>
<tr>
<td>UMSCC-103a</td>
<td>F</td>
<td>Lateral tongue</td>
<td>T4N2bMx</td>
</tr>
</tbody>
</table>

Abbreviations: BOT, base of tongue; FOM, floor of mouth; HNSCC, head and neck squamous cell carcinoma.

aUMSCC-103 was derived from primary tumor HN-111.
and CD44high cells were resuspended in DMEM containing 1% fetal bovine serum, 1% penicillin-streptomycin, and 1% nonessential amino acids. Equal numbers of sorted cells were plated in the upper wells of Matrigel-coated and control chambers (BD BioCoat Matrigel Invasion Chamber; BD Biosciences, San Jose, California), with DMEM containing 10% fetal bovine serum and 30-ng/mL epidermal growth factor (human recombinant; Sigma-Aldrich, St Louis, Missouri) serving as the chemoattractant in the lower well. The chambers were incubated for 24 to 48 hours at 37°C, with the duration dependent on the cell line. After incubation, cells remaining in the upper well were removed by scrubbing twice with a cotton-tipped swab, and cells that had migrated into the lower well were fixed and stained with crystal violet in 20% methanol for 30 minutes. The chambers were then washed twice in deionized water and allowed to dry. The dried stain was dissolved in 10% acetic acid, and the solution from each chamber was transferred to a 96-well plate. Invasion was then quantified by measuring the absorbance at 560 nm. Control chambers were used to assess motility, while Matrigel chambers served as models for basement membrane invasion (Figure 4A and Figure 5). Assays for all cell lines were performed in duplicate or triplicate, with the exception of UMSCC-103.

RESULTS

IN VITRO MODEL OF METASTASIS

Boyden migration chambers have served as a relatively simple and inexpensive in vitro model of invasion for over a decade. These chambers consist of an upper and a lower well. In the control chambers, the upper and lower wells
are separated by a polycarbonate filter with 8-µm pores. Only cells with sufficient motility can migrate into the lower well of the chamber. In the Matrigel-coated chambers, there is a thin layer of Matrigel covering the upper surface of the polycarbonate filter.\(^8\) Composed of extracellular matrix proteins, this gel layer serves as an analog for the basement membrane. Because the basement membrane is a barrier between epithelial or endothelial cells and the underlying stroma, the ability to invade through this line of defense is a key step in the metastatic process. Matrix metalloproteinases, integrins, and other matrix receptors are known to be essential in this pathologic step. CD44, a hyaluronan receptor, has been shown to mediate invasion in both melanomas and gliomas.\(^9,10\)

Cells expressing the highest levels of CD44 and those expressing little to no CD44 were collected using fluorescence-activated cell sorting (Figure 2). Control chambers were used to quantify general motility of CD44\(^ {\text{high}}\) and CD44\(^ {\text{low}}\) populations, and Matrigel-coated chambers were used to quantify invasion. Interestingly, for all but one of the cell lines studied, the CD44\(^ {\text{high}}\) cells did not invade through the Matrigel more efficiently than the CD44\(^ {\text{low}}\) cells. Moreover, for many of the cell lines, there was a trend toward the CD44\(^ {\text{low}}\) cells being more invasive (Figure 4A and Figure 5). The only exception was UMSCC-12, derived from a laryngeal squamous cell carcinoma, for which the CD44\(^ {\text{high}}\) cells were both more motile and more invasive (Figure 4A and Figure 5). The only exception was UMSCC-12, derived from a laryngeal squamous cell carcinoma, for which the CD44\(^ {\text{high}}\) cells were both more motile and more invasive. In contrast, the CD44\(^ {\text{high}}\) cells migrated through the control chambers more efficiently in almost all cell lines and, therefore, proved to be more motile than their CD44\(^ {\text{low}}\) counterparts (Figure 4B and Figure 5). UMSCC-14B, which arose from a recurrent squamous cell carcinoma of the floor of mouth, was the only cell line in which low CD44 expression correlated with better motility.

**IN VIVO MODEL OF METASTASIS**

To study the effect of CD44 expression on metastasis in a more nuanced environment, an animal model of lung colonization was used. The NOD-SCID mice were injected with either CD44\(^ {\text{high}}\) or CD44\(^ {\text{low}}\) cells via the tail vein. The presence of lung lesions was assessed either via necropsy and histologic examination 6 months after injection (older method) or via luciferase-mediated bioluminescence 2 months after injection (newer method). With the use of the older method, 3 of 4 mice injected with CD44\(^ {\text{high}}\) cells developed metastases, while 0 of 4 injected with CD44\(^ {\text{low}}\) cells had lung lesions. Notably, the cell suspension derived from a primary tumor formed distant lesions in 2 of 2 mice after injection with only 1 \(\times 10^4\) cells, while the established cell line UMSCC-6 grew a lung lesion in 1 of 2 mice after injection of 5 \(\times 10^4\) cells (Table 2).

**Table 2. CD44 Expression and Lung Colonization**

<table>
<thead>
<tr>
<th>CD44(^ {\text{high}})</th>
<th>CD44(^ {\text{low}})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. Mice Injected</strong></td>
<td><strong>No. Lung Lesions</strong></td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>HN-111(^b), 1 (\times 10^4) cells</td>
<td>2</td>
</tr>
<tr>
<td>UMSCC-6, 5 (\times 10^4) cells</td>
<td>2</td>
</tr>
<tr>
<td>UMSCC-47, 5 (\times 10^4) cells</td>
<td>6</td>
</tr>
<tr>
<td>UMSCC-14B, 3 (\times 10^4) cells</td>
<td>2</td>
</tr>
<tr>
<td>UMSCC-12, 5 (\times 10^4) cells</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>17</td>
</tr>
</tbody>
</table>

\(^a\)CD44\(^ {\text{high}}\) and CD44\(^ {\text{low}}\) cells from a primary tumor (HN-111) and 4 cell lines were injected via the tail vein into NOD/SCID mice. Lungs were evaluated via sectioning or bioluminescence imaging. CD44\(^ {\text{high}}\) cells formed lung lesions 8 of 17 times vs 0 of 17 times by CD44\(^ {\text{low}}\) cells.

\(^b\)HN-111 later became the cell line UMSCC-103.

Figure 3. In vivo model of metastases. Bioluminescence imaging of nonobese diabetic severe combined immunodeficient mice 8 weeks after tail vein injection with UMSCC-47 cells transduced with luciferase. Left, injected with 5 \(\times 10^4\) cells with low CD44 expression; middle, injected with 5 \(\times 10^4\) cells with high CD44 expression; and right, no injection. Min indicates minimum; Max, maximum; and sr, steradian.

![Image](https://example.com/image.png)
A total of 14 mice were injected with UMSCC-47-Luc, 7 with CD44\textsuperscript{high} cells, and 7 with CD44\textsuperscript{low} cells. Four injections were performed at an earlier date (group A), and 10 were performed later (group B). Unfortunately, 2 of the mice from group A were not evaluable. Group A was observed for 6 months, while group B was observed for 2 months. Two of the mice injected with UMSCC-47-Luc CD44\textsuperscript{high} cells (1 from each group) developed lung lesions, while the injections with CD44\textsuperscript{low} cells did not produce any signs of disease (Figure 3). The lung lesions from the mouse in group A were dissected and cultured in vitro (Figure 1A and B). The cells derived from this lung lesion were then analyzed for CD44 expression using flow cytometry. This analysis revealed that the CD44\textsuperscript{high} cells originally injected into the mouse had reconstituted a heterogeneous population of cells with both high and low CD44 expression (Figure 1C and D).

Four mice were injected with UMSCC-14B-Luc cells. After 6 months, UMSCC-14B-Luc tail vein injections produced lung lesions in 1 of 2 mice injected with CD44\textsuperscript{high} cells and 0 of 2 mice injected with CD44\textsuperscript{low} cells. A total of 10 mice were injected with UMSCC-12-Luc cells, 5 with CD44\textsuperscript{high} cells, and 5 with CD44\textsuperscript{low} cells. After 2 months of observation, lung lesions developed in 2 of 5 mice injected with CD44\textsuperscript{high} cells. One of these mice died prior to being imaged with Bioluminescent imaging and was instead evaluated by necropsy. No lesions were seen in the mice injected with CD44\textsuperscript{low} cells. Overall, CSCs gave rise to lung lesions in 8 of 17 mice (47%), while non-CSCs did not produce any distant disease (Table 2).

**COMMENT**

The finding that CSCs, as identified by high CD44 expression, were not more invasive than non-CSCs in vitro could be attributed to many factors. First, it may be owing to shortcomings of the experimental model, which is a simplified representation of a complex system. Although Matrigel invasion chambers have been used in many experimental designs, they only represent some of the first steps in a long chain of events required for a cell to successfully metastasize. As Paget described in the 19th century, the process of metastasis follows a seed-and-soil model. The cells (“seeds”) need to have the appropriate mechanisms in place to dissociate from the primary tumor, enter into the lymphatics or bloodstream, and escape the circulation to find a new home. In addition, the site of metastasis (“soil”) must be properly suited to signal to the circulating cells and allow a new tumor to form from them.12 This is often referred to as the tumor microenvironment, and its key attributes are still poorly understood.

While our model used epidermal growth factor and serum as chemoattractants, which are both commonly described in the literature, perhaps these are not the signaling elements that entice an HNSCC cancer cell to metastasize in vivo. Matrigel is largely composed of laminin, collagen IV, and heparan sulfate proteoglycans.13 Since CD44 is primarily a receptor for hyaluronan, it seems plausible that Matrigel is not an appropri-

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**Figure 4.** Matrigel invasion and control migration chamber results. A, Matrigel invasion chamber results. Invasion was quantified by staining cells in the lower well of the chamber and measuring absorbance from each chamber at 560 nm. Absorbances for chambers containing CD44\textsuperscript{high} vs CD44\textsuperscript{low} were compared. B, Control migration chamber results. Motility was quantified by staining cells in the lower well of the chamber and measuring absorbance from each chamber at 560 nm. Absorbances for wells containing CD44\textsuperscript{high} vs CD44\textsuperscript{low} were compared. Error bars indicate standard deviations.

**Figure 5.** Differences in invasion and migration for CD44\textsuperscript{high} vs CD44\textsuperscript{low} cells. Differences were calculated as % change\textsuperscript{invasion} = [(A560 nm for CD44\textsuperscript{high}) − (A560 nm for CD44\textsuperscript{low})] / (A560 nm for CD44\textsuperscript{low}) × 100%. Invasion was measured using Matrigel-coated chambers, while migration was calculated using control (uncoated) chambers. Negative values for % change reflect more efficient migration or invasion by CD44\textsuperscript{low} cells.
ate model for studying this surface protein. Draffin et al.14 studied in vitro invasion of 2 prostate cancer cell lines, one with and one without CD44 expression, using Matrigel invasion chambers. Although the CD44+ cell line showed a significant increase in invasion when Matrigel chambers were supplemented with hyaluronan, the CD44+ cell line invaded more efficiently when chambers were not supplemented.

The lack of correlation between high CD44 expression and invasion may also be related to the limitations of using a single-cell marker to identify CSCs. Previous studies of CSCs and metastasis in other tumor types have used 2 or 3 markers to identify the metastatic subset.6,7 It is likely that the same is true for head and neck cancer stemlike cells. Metastasis formation is a complex process and, although CD44 may mediate 1 or 2 pivotal steps in this series of events, it is plausible that there are additional crucial cell characteristics. For example, the relatively poor ability of CD44high cells to invade through the basement membrane may represent low expression of matrix metalloproteinases in these cells.

In contrast, the results from our animal model strongly suggest that HNSCC stemlike cells have enhanced metastatic potential. The somewhat contradictory results between our models are likely due to their significant differences in design and intricacies. Notably, the duration of the in vivo experiments was much longer than that of the in vitro assays. This time lapse could have allowed the CD44high cells to alter their expression profiles significantly, such that they expressed factors necessary for invasion out of the bloodstream and into the tissues. It should be noted that while lung colonization is a commonly used model for studying the processes involved in metastases, it has significant limitations. Most notably, HNSCC metastases typically form via lymphatic spread, and the lung colonization model does not account for certain characteristics required of cells for entry into the lymphatics. While many steps in the formation of metastases are common to both lymphatic and hematogenous spread, there are likely key differences. The epithelial-to-mesenchymal transition (EMT) is well described in the embryology literature; in addition, it is thought to mediate invasion of cancer cells into the surrounding stroma. Epithelial-to-mesenchymal transition occurs when, in response to transforming growth factors or other signals, cells dissociate from one another, lose expression of epithelial markers and gain expression of mesenchymal ones, alter their polarization and cytoskeletal structure, and establish new cell-matrix interactions. A similar process is required of cancer cells that are destined to metastasize.15

The increased motility seen in CD44high cells is characteristic of cells undergoing EMT, and this may explain why, in our study, head and neck CSCs formed lung lesions in vivo, while non-CSCs did not. In fact, Takahashi et al.16 showed that, in EMT induced by tumor necrosis factor, the interaction between CD44 and hyaluronan indeed mediated cell-cell dissociation, actin remodeling, and, as a result, enhanced motility. These findings, in conjunction with our own, suggest that cell motility and the ability to undergo EMT are some of the most important characteristics of a metastatic cell, and it appears that CSCs may have those capabilities.

Future studies focused on better understanding the role of CSCs in EMT as it relates to HNSCC are needed. In addition, further purification of the stemlike cell population in HNSCC is necessary to clarify what metastatic characteristics are indeed unique to these cells. Such knowledge would allow clinicians to exploit this particular set of attributes to target cancer cells that keep a tumor growing and allow it to spread.

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Author Contributions: The principal investigators Ms Davis and Drs Carey and Prince had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Davis, Divi, Owen, Carey, Papagerakis, and Prince. Acquisition of data: Davis, Divi, Owen, Papagerakis, and Prince. Analysis and interpretation of data: Davis, Owen, Bradford, Carey, Papagerakis, and Prince. Drafting of the manuscript: Davis, Papagerakis, and Prince. Critical revision of the manuscript for important intellectual content: Davis, Divi, Owen, Bradford, Carey, Papagerakis, and Prince. Obtained funding: Papagerakis and Prince. Administrative, technical, and material support: Owen and Bradford. Study supervision: Davis, Owen, Bradford, Carey, Papagerakis, and Prince.

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