Metastatic Potential of Cancer Stem Cells in Head and Neck Squamous Cell Carcinoma

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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^high cells were more motile but not more invasive than CD44^low cells. In vivo, 8 of 17 mice injected with CD44^high cells and 0 of 17 mice injected with CD44^low 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.


Worldwide, malignant neoplasms of the head and neck account for approximately 500,000 new cases of cancer annually.1 Despite an ever-expanding fund of knowledge regarding the etiology and pathophysiology of malignant neoplasms, head and neck squamous cell carcinoma (HNSCC) continues to be a disfiguring and deadly disease. For patients with squamous cell carcinoma of the oral cavity or oropharynx, the 5-year survival is a dismal 56%, which has remained relatively unchanged in recent years.2 This poor prognosis reflects the fact that most patients present with advanced-stage disease, often making a complete cure a seemingly unattainable goal. In fact, just 46% of oral cavity and 16% of oropharyngeal cancers are diagnosed when there is only local disease.2

Subpopulations of highly tumorigenic cells, or cancer stem cells (CSCs), have been identified in multiple tumor types, both solid and hematologic, using a variety of cell markers.3 These cells have the unique capacity to self-renew and produce differentiated progeny. These characteristics allow CSCs to maintain a pluripotent phenotype, while also producing a tumor composed of a heterogeneous cell population. In HNSCC, the CSC population is contained within the cell fraction that expresses high levels of the surface glycoprotein CD44.4 This transmembrane protein serves as a receptor for hyaluronan and, to a lesser extent, for other extracellular matrix components, and its function can be altered with alternative splicing and glycosylation.5 In HNSCC, cells expressing high levels of CD44 have primitive morphologic features, express high levels of the stem cell marker BMI-1, and co-stain with cytokeratin 5/14, a basal cell marker. In vivo, CD44^high cells are capable of regenerating a heterogeneous tumor, whereas their CD44^low counterparts cannot.4

Although CSCs are known to exhibit increased tumorigenicity compared with the rest of the tumor population, it is largely unknown what role they play in local invasion and distant metastatic spread. Because most patients with cancer die of metastatic disease, it is crucial to elucidate the mechanisms by which cancers gain access to and seed distant tissues. In breast cancer, it has been shown that approximately 30% of patients have occult...
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 backbone and a cytomegalovirus promoter. Polybrene was added to increase efficiency of the transduction. Successful gene delivery was confirmed via green fluorescent protein (GFP) visualization in a side-by-side transduction of the HIV-GFP vector under identical conditions.

PRIMARY TUMOR DIGESTION

Tumors were cut into small fragments, further minced with a sterile scalpel, and then placed in a solution of Media 199 and 200-U/mL collagenase III. The mixture was incubated at 37°C for up to 3 hours to allow complete digestion. Every 15 minutes, the solution was mixed through a 10-mL pipette to encourage dissociation. Cells were filtered through 40-µm nylon mesh and washed twice with Hank's Balanced Salt Solution containing 2% heat-inactivated calf serum (HBSS–2% HICS), then stained for flow cytometry.

FLOW CYTOMETRY

Cells were detached from tissue culture-treated flasks using a 0.125% trypsin solution with EDTA. The single-cell suspensions were washed in HBSS–2% HICS, counted, and then resuspended to a concentration of 10^6 cells per 1-mL HBSS/2%

| Table 1. Characteristics of HNSCC Cell Lines |
|----------------|----------------|----------------|----------------|
| Cell Line | Patient Gender | Specimen Site | TNM Stage |
| UMSCC-6 | M | BOT | T2N0M0 |
| UMSCC-10A | M | True vocal cord | T3N0M0 |
| UMSCC-12 | M | Larynx | T2N1M0 |
| UMSCC-14A | F | FOM | T1N0M0 |
| UMSCC-14B | F | FOM | T1N0M0 |
| UMSCC-47 | M | Lateral tongue | T3N1M0 |
| UMSCC-103 | M | Lateral tongue | T4N2bM1 |

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

HICS. The suspensions were then incubated with either anti-CD44 antibody (allopurinol-conjugated, mouse antihuman, clone G44-26; BD Pharmingen, San Diego, California) or mouse IgG2b, κ isotype control antibody (allopurinol-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, -CD64, 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 10^6 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 1C and D). The 10% to 15% of cells with the highest and lowest CD44 expression were collected for use (Figure 2).

TAIL VEIN INJECTIONS

CD44<sup>high</sup> and CD44<sup>low</sup> cells from HN-111, UMSCC-6, UMSCC-47-Luc, UMSCC-12-Luc, and UMSCC-14B-Luc were reseeded in phosphate-buffered saline to a concentration of 2.5 × 10<sup>6</sup> cells/mL (for cell lines) or 5.0 × 10<sup>6</sup> cells/mL (for primary tumor cells). A total volume of up to 200 µL 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).

BIOLUMINESCENT 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 µL 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. CD44<sup>high</sup>
and CD44<sub>low</sub> cells were resuspended in DMEM containing 1% fetal bovine serum, 1% penicillin-streptomycin, and 1% non-essential 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 chemotactant 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

Figure 1. Lung lesion from UMSCC-47 CD44<sup>high</sup> cells. A, Hematoxylin-eosin–stained section of lung lesion (original magnification ×10). B, Lung nodule cultured in vitro (original magnification ×20). C, Fluorescence-activated cell sorting plot for lung lesion cells stained with CD44 isotype control antibody. D, Fluorescence-activated cell sorting plot for lung lesion cells stained with antihuman CD44 antibody. The CD44<sup>high</sup> cells reconstituted a heterogeneous population, with 41.4% of the lung lesion cells expressing CD44. APC-A indicates allophycocyanin; SSC-A, side scatter.

Figure 2. CD44 expression in head and neck squamous cell carcinoma cell lines. Each bar represents the percentage of the cell population with CD44 expression, with the threshold for expression defined by staining with an isotype control antibody. The 10% to 15% of cells with the highest expression were collected as CD44<sup>high</sup>, and the 10% to 15% with the lowest or no expression were collected as CD44<sup>low</sup> cells.

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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. 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.

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 CD44high and CD44low populations, and Matrigel-coated chambers were used to quantify invasion. Interestingly, for all but one of the cell lines studied, the CD44high cells did not invade through the Matrigel more efficiently than the CD44low cells. Moreover, for many of the cell lines, there was a trend toward the CD44low 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 CD44high cells were both more motile and more invasive. In contrast, the CD44high cells migrated through the control chambers more efficiently in almost all cell lines and, therefore, proved to be more motile than their CD44low 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 CD44high or CD44low 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 imaging 2 months after tail vein injection with UMSCC-47-Luc cells transduced with luciferase. Left, injected with 5 × 10⁶ cells with low CD44 expression; middle, injected with 5 × 10⁴ cells with high CD44 expression; and right, no injection. Min indicates minimum; Max, maximum; and sr, steradian.

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

<table>
<thead>
<tr>
<th>CD44&lt;sup&gt;high&lt;/sup&gt;</th>
<th>CD44&lt;sup&gt;low&lt;/sup&gt;</th>
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<tbody>
<tr>
<td><strong>No. Mice Injected</strong></td>
<td><strong>No. Lung Lesions</strong></td>
</tr>
<tr>
<td>HN-111&lt;sup&gt;b&lt;/sup&gt;, 1 × 10⁴ cells</td>
<td>2</td>
</tr>
<tr>
<td>UMSCC-6, 5 × 10⁴ cells</td>
<td>2</td>
</tr>
<tr>
<td>UMSCC-47, 5 × 10⁴ cells</td>
<td>5</td>
</tr>
<tr>
<td>UMSCC-12, 5 × 10⁴ cells</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>17</td>
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<sup>a</sup>CD44<sup>high</sup> and CD44<sup>low</sup> 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<sup>high</sup> cells formed lung lesions 8 of 17 times vs 0 of 17 times by CD44<sup>low</sup> cells.

<sup>b</sup>HN-111 later became the cell line UMSCC-103.
A total of 14 mice were injected with UMSCC-47-Luc, 7 with CD44high cells, and 7 with CD44low 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 CD44high cells (1 from each group) developed lung lesions, while the injections with CD44low 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 CD44high 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 CD44high cells and 0 of 2 mice injected with CD44low cells. A total of 10 mice were injected with UMSCC-12-Luc cells, 5 with CD44high cells, and 5 with CD44low cells. After 2 months of observation, lung lesions developed in 2 of 5 mice injected with CD44high 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 CD44low 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-
ate model for studying this surface protein. Draffin et al\(^1\) 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 CD44\(^{high}\) 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 CD44\(^{high}\) 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 CD44\(^{high}\) 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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