ERK1/2 Regulation of CD44 Modulates Oral Cancer Aggressiveness

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Abstract
Carcinogen-induced oral cavity squamous cell carcinoma (OSCC) incurs significant morbidity and mortality and constitutes a global health challenge. To gain further insight into this disease, we generated cell line models from 7,12-dimethylbenz(a)anthracene–induced murine primary OSCC capable of tumor formation upon transplantation into immunocompetent wild-type mice. Whereas several cell lines grew rapidly and were capable of metastasis, some grew slowly and did not metastasize. Aggressively growing cell lines displayed ERK1/2 activation, which stimulated expression of CD44, a marker associated with epithelial to mesenchymal transition and putative cancer stem cells. MEK (MAP/ERK kinase) inhibition upstream of ERK1/2 decreased CD44 expression and promoter activity and reduced cell migration and invasion. Conversely, MEK1 activation enhanced CD44 expression and promoter activity, whereas CD44 attenuation reduced in vitro migration and in vivo tumor formation. Extending these findings to freshly resected human OSCC, we confirmed a strict relationship between ERK1/2 phosphorylation and CD44 expression. In summary, our findings identify CD44 as a critical target of ERK1/2 in promoting tumor aggressiveness and offer a preclinical proof-of-concept to target this pathway as a strategy to treat head and neck cancer. Cancer Res; 72(1); 365–74. © 2011 AACR.

Introduction
Oral cavity squamous cell carcinoma (OSCC) is a prominent subset of head and neck cancers, which are the sixth most common cancer worldwide (1). The major risk factor for developing OSCC is carcinogen exposure, which distinguishes this subset of head and neck cancers from those induced by human papillomavirus (HPV; ref. 1, 2). Despite advances in detection, surgery, chemotherapy, and radiation, the prognosis for OSCC has remained stable for decades (1–3). Furthermore, at the time of diagnosis, approximately two-thirds of OSCC patients have locoregionally advanced disease resulting in increased morbidity and mortality (1–3).

Multiple mutations and epigenetic alterations of signaling and regulatory proteins have been identified as promoters of OSCC. These include alterations in TP53, CDKN2A, PTEN, PIK3CA, and Notch1 (4, 5). The RAS signaling pathway is also modulated, with 5% to 50% of OSCC having mutated RAS and 45% to 80% having overexpressed nonmutated RAS (1, 6). Similarly, overexpression of the epidermal growth factor receptor (EGFR), found in 80% to 90% of OSCC, heralds a worse prognosis (7). Furthermore, key progrowth and prosurvival signaling cascades, including STAT3, protein kinase B (AKT), and NF-κB, are constitutively activated in OSCC (8). The extracellular signal-regulated kinases (ERK1/2) have received less attention in OSCC but do activate interleukin 8 and VEGF expression in human OSCC cell lines and, in the context of PTPN13 loss, promote tumorigenesis in both HPV-positive and HPV-negative squamous cancers (9, 10). Alterations in these pathways are common to many OSCC, and thus, defining pathways or tumor subtypes that distinguish more aggressive lesions is critical for therapeutic targeting.

CD44+ cells are such targets for 3 reasons—first, they have enhanced chemoresistance; second, they are proposed to be cancer stem cells (CSC) as they engraft at higher frequencies in mice; and third, in other systems, they are associated with the epithelial to mesenchymal transition (EMT), a genetic program associated with metastasis (11–13). CD44 functions as a transmembrane protein that, along with RHAMM, is the principal receptor for hyaluronan and also is a coreceptor for several receptor tyrosine kinases, including c-MET and EGFR (14, 15). Initial work on its regulation focused on alternative splicing and showed that the v6 CD44 isoform promoted metastatic activity (16). In addition, other levels of regulation have been shown including p53-mediated repression, RAS-mediated alternative splicing or transcription, and microRNA-mediated attenuation (17–20). Regulation of CD44 expression remains

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incompletely understood in head and neck cancers. In OSCC, increased CD44 expression has been associated with decreased survival and increased recurrence, metastasis, and resistance to chemo/radiation therapy (11, 21, 22). Whether these properties are due to a functional contribution of CD44 or whether CD44 is simply a marker of cells with more aggressive behavior has not been fully explored in OSCC (21).

Models for the study of OSCC have relied primarily on immunodeficient xenograft models, which overlook tumor–immune interactions. In contrast to the extensive literature on syngeneic murine transplantable models in other tumor systems, studies in OSCC are limited. Herein, we describe a transplantable syngeneic murine model of OSCC derived from carcinogen-induced primary tumors. Utilizing this model, we identified that increased activation of ERK1/2 was associated with locoregional aggressiveness and enhanced transcription of CD44, a key driving force for in vitro migration and in vivo growth. We then extended these data to human OSCC and identified that increased ERK1/2 phosphorylation and CD44 expression were tightly associated. Thus, these mouse cell lines provide insight into ERK1/2–CD44 contribution to tumor aggressiveness and constitute a syngeneic, transplantable murine model of OSCC that will allow an evaluation of the intrinsic tumor biology and the host immune responses to OSCC development and metastasis.

Materials and Methods

Animals

C57BL/6 mice were from Taconic and CXCR3−/− mice have been described (23). Studies were done under approved protocols of the Animal Studies Committee of Washington University.

Plasmids

Mouse CD44 short hairpin RNA (shRNA) and control scrambles were from Sigma-Aldrich. Mouse CD44 luciferase reporters were provided by Mark Perrella (24). The estrogen regulated constitutively active MEK1/R4F-ER was from Addgene (25).

Antibodies

All primary antibodies were from Cell Signaling Technologies except anti-STAT3 (Santa Cruz Biotechnology) and anti–β-actin (Sigma-Aldrich). Secondary antibodies were conjugated to Alexa Fluor 680 (Invitrogen) or IRDye 800 (Rockland). PE–anti-mouse/human CD44 (IM7), PE-rat IgG2b–κ isotype control, and APC anti-mouse CD24 were from Biolegend. For immunofluorescence, anti-cytokeratin (Dako), CD44 (IM7; BD Biosciences), and p-ERK1/2 (Cell Signaling Technologies) were used. Secondaries were Cy2 and Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch), and Alexa-488 goat anti-rat antibodies (Invitrogen).

Cell lines

Primary 7,12-dimethylbenz(a)anthracene (DMBA)-induced mouse OSCCs were generated as described (26). Single-cell suspensions of individual primary oral cavity tumors were made with Collagenase IA (Sigma-Aldrich) and cultured in IMDM/F12 (2:1) with 5% fetal calf serum, penicillin/streptomycin, 1% amphotericin, 5 ng/mL EGF (Millipore), 400 ng/mL hydrocortisone, and 5 μg/mL insulin. Sequential differential trypsinization was then used to clear fibroblast contamination. MOC1, 7, 10, 22, and 23 were derived from primary tumors in C57BL/6 mice, and MOC2 was derived from a chemokine receptor CXCR3-deficient mouse on a pure C57BL/6 background (ref. 27; of note, no major differences in the incidence of tumor formation were noted between the different genotypes). CXCR3 is not detectable on oral keratinocytes and does not contribute to MOC2 growth (Supplementary Fig. S3). Immunofluorescence staining for cytokeratin was done to confirm an epithelial phenotype (Fig. 1C and Supplementary Fig. S1C). PCI-13 was obtained from Dr. Theresa Whiteside, UPCI: SCC029B and UPCI:SCC068 were obtained from Dr. Suzanne Gollin, and all were used with minimal passaging. The UM-SCC-1 cell line (from Dr. Tom Carey) was genotyped in May, 2011 and concordance with published data was established (27).

Transwell migration assay

A total of 1 × 105 cells were loaded into the upper chamber and complete media in the lower chamber of Transwell plates (8 μm; BD Biosciences). After incubation for 24 hours, cells in the lower chamber were fixed and stained (DiffQuick; Dade Behring) and counted.

Wound healing (scratch) test

Cells at 80% confluency were wounded with a sterile 200 μL pipette. Cell migration was recorded by microscopy at 0 and 24 hours.

Western blot and immunoprecipitation

Cell Extraction Buffer (Invitrogen) was used to make lysates and Western blots were carried out as described (28). A Pierce Coimmunoprecipitation Kit (Thermo Scientific) was used for STAT3 analysis.

Fluorescence-activated cell sorting

Tumor cells were blocked with rat serum and stained with antibodies at 4°C for 30 minutes. Data were collected on a FACSCalibur (BD Biosciences) and analyzed using Flojo software (Tree Star). Further details are included in the Supplementary Methods.

Immunofluorescence

Cells were grown on cover slips, fixed, and permeabilized. Paraffin embedded sections underwent antigen retrieval. Specimens were blocked and then incubated with primary antibodies for 1 hour. Detection was accomplished with secondaries and 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) for the nuclear stain.

Tumor transplantation

Cell lines were harvested, washed twice in D-PBS (Fisher), and injected into the right subcutaneous flank of mice. Tumor growth was recorded as the average of the 2 largest diameters.
Luciferase assay and infections

Cell lines were transfected (Fugene; Promega) and lysates were analyzed using the Dual Luciferase Reporter System (Promega). For shRNA targeting, cell lines underwent lentiviral transduction and selected using puromycin (2.5 μg/mL). For analysis of enforced ERK1/2 activation, MEK1/R4F-ER was retrovirally transduced into MOC1. Cells were then treated with vehicle or 4-OH-tamoxifen (200 nmol/L, referred to as tamoxifen) for 48 hours and analyzed for CD44 expression. CD44 luciferase activity was evaluated 24 hours after cotransfection with MEK1/R4F-ER and CD44 reporter. For studies with U0126 (10 μmol/L), cell lines were transfected and treated with drug for 24 hours and assays done as above.

Human tumor analysis

Under an Institutional Review Board–approved protocol at Stanford University, human primary OSCC tumors were dissociated and analyzed by flow cytometry. CD45+/CD31− cells were excluded and tumor cells analyzed for CD44 and phospho-ERK1/2 (see Supplementary Methods).

Statistical analysis

All analyses were done with oversight from a biostatistician. Heterotopic tumor growth was analyzed by single-day comparison analysis using Mann–Whitney U test (Nonparametric equivalent of independent samples t test). Migration differences (Fig. 2D) were analyzed by independent samples t test, and in addition, a 2-way ANOVA was carried out. For comparison of tumor growth in CD44 knockdowns (Fig. 3E), a mixed between–within subjects ANOVA comparing scramble with the indicated CD44 knockdown was done. Luciferase assays were all analyzed using independent samples t tests as indicated.

Results

A new OSCC model

To model OSCC, we generated 6 transplantable mouse OSCC cell lines from independent carcinogen-induced tumors (26). After 25 weeks of twice weekly oral cavity DMBA application, mice developed SCCs (Fig. 1A and B and Supplementary Fig. S1A and B) and 6 cell lines, designated mouse oral cancer (MOC1, 2, 7, 10, 22, and 23), were derived. To assess their in vivo growth, cell lines were transplanted either heterotopically in the flank, where tumors can be easily monitored, or orthotopically in the floor of mouth/buccal region (Supplementary Fig. S2B). Growth was seen at both sites; thus for ease of measurement, we utilized heterotopic transplantation for these studies. The cell lines segregated into either an indolent (MOC1, 22) or aggressive (MOC2, 7, and 10) growth phenotypes (Fig. 1D and Supplementary Fig. S2A). MOC23 did not form tumors in WT mice and only grew in RAG2−/− mice, as this line does not form progressive tumors in WT mice. A representative primary OSCC in left floor of mouth/buccal region after 25 weeks of twice weekly DMBA treatment (MOC10 parent tumor). B, representative H&E-stained section of primary tumor (MOC10 parent tumor) showing moderately differentiated squamous cell carcinoma (40× magnification). C, immunofluorescence of MOC10 cell line showing epithelial phenotype with positive cytokeratin staining (see Supplementary Figure S1C) (40× magnification). D, representative in vivo growth curves comparing indolent MOC1 and aggressive MOC2 after 1 × 106 cells were injected into the right flank of C57BL/6 WT mice (**, P < 0.01 in all days post day 0). E, metastatic inguinal draining lymph node (black filled arrow) that is enlarged and discolored compared with contralateral normal appearing lymph node (black open arrow). F, H&E-stained metastatic lymph node shows effacement of normal architecture by SCC (LN = lymph node, 20× magnification). G, summary of LN metastatic capacity of each cell line. (MOC23 data is shown for RAG2−/− mice, as this line does not form progressive tumors in WT mice). H&E, hematoxylin and eosin stain.

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RAG2<sup>−/−</sup> immunodeficient mice (data not shown). The aggressive growth of MOC2 and MOC10 was illustrated by their ability to form tumors with injection of as few as 10,000 cells (Fig. 3E and Supplementary Fig. S6E). Notably, the cell lines reflected the clinical appearance of the primary tumors—MOC1, 22, and 23 were derived from exophytic lesions (see Supplementary Fig. S1A for MOC1), whereas MOC2, 7, and 10 were invasive appearing (Fig. 1A and Supplementary Fig. S1A).

**Lymph node metastatic capacity of MOC cell lines**

A key poor prognosticator of human OSCC is the presence of lymph node (LN) metastases. To assess this in the MOC lines, Vehicle U0126 BA

![Graph](image)

**Figure 2.** Increased activation of ERK1/2 is associated with increased OSCC aggressiveness. A, Western blots of MOC1/2/7/10 for phospho-NF-κB, NF-κB, phospho-AKT, AKT, EGFR, TGFβR, and β-actin. STAT3 and phospho-STAT3 were visualized by immunoprecipitation and Western blotting. Note in the experiment shown MOC7 had less total STAT3 immunoprecipitated, but in repeat experiments it was found to express similar amounts of STAT3 when compared with the other cell lines (data not shown). B, Western blot of p-ERK1/2 and ERK1/2 for all 6 MOC lines. C, representative 20× microscopic image of a Transwell migration assay of MOC2 cells treated with vehicle control (DMSO) or U0126 (10 μmol/L). D, quantitation of Transwell migration assay where 4 random sections per filter × 3 filters were counted in a blinded fashion by light microscopy at 20× magnification for both MOC1 and MOC2 cells treated with vehicle or U0126 (**P < 0.001). The percentage decrease relative to vehicle treatment is indicated above the bar graph. DMSO, dimethyl sulfoxide.

![Graph](image)

**Figure 3.** CD44 is associated with and contributes to increased OSCC aggressiveness. A, FACS analysis of cell surface CD44 expression by pan-CD44 antibody (IM7) and isotype control shown for MOC1, 2, and 10. B, representative mean fluorescence intensity for cell surface CD44 expression in all 6 cell lines (from one of at least 3 experiments). C, FACS analysis of cell surface CD44 expression in MOC10 after shRNA knockdown of CD44 with 3 distinct shRNAs (CD44-6, CD44-7, and CD44-10) or scramble control shRNA (quantitated by indicated MFI). D, scratch test of MOC10 after transduction with indicated CD44 or scramble shRNAs. E, CD44 shRNA knockdown leads to delayed or abrogated growth of MOC10. MOC10 cells (1 × 10<sup>5</sup>) transduced with scramble, CD44-7, or CD44-10 shRNA were injected into the right flank of C57BL/6 WT mice and monitored. Growth curves only represent average tumor diameter of transplanted tumors that grew out as indicated (**P < 0.001), MFI, mean fluorescence intensity.
tissues from all mice in the transplant experiments in Fig. 1D and Supplementary Fig. 5A were examined for metastases (Fig. 1E–G). We did not observe any lung or liver metastases in these mice, but interestingly, MOC2/7/10 displayed spontaneous metastasis to regional LNs in 5/5, 4/5, and 5/5 mice, respectively. Orthotopic injection of MOC2 and 10 also showed cervical metastasis at the same rate as in the flank (Supplementary Fig. S2C). In contrast, MOC1 and MOC22 did not metastasize regionally or distantly (combined n = 30 for MOC1 and n = 10 for MOC22). When 1 x 10^4 MOC10 cells were injected, slower but progressive primary tumor growth was seen and both lymphatic and lung metastases were found (Supplementary Fig. S2D and E), suggesting that distant metastases require a longer primary tumor growth. Thus, 3 of 6 lines displayed regional LN metastasis recapitulating an important aspect of human OSCC.

**ERK1/2 activation is associated with increased OSCC aggressiveness**

Given differences in growth phenotypes, we next addressed possible aberrant intracellular signaling pathways that could mediate these differences. We focused on well-characterized pathways from human OSCC and found minimal correlation in levels of EGFR, TGFβRII, phospho-AKT (Ser473), phospho-NFκB p65 (Ser536), or phospho-STAT3 (Tyr705) with the aggressive phenotype (Fig. 2A). Interestingly, the most profound difference was in the level of phosphorylated ERK1/2 (Thr202/Tyr204). MOC2/7/10, which all grew aggressively and consistently metastasized to draining lymph nodes in vivo, had increased phospho-ERK1/2 compared with the indolent MOC1, 22, or 23 (Fig. 2B).

**K-RAS mutations are associated with increased phospho-ERK1/2 in MOC lines**

Although DMBA is widely mutagenic, DMBA-induced RAS family mutations are key oncogenic drivers in multistage carcinogenesis (29). To this end, we evaluated H-, K-, N-RAS, and B-Raf for mutations in the cell lines. We found no N-RAS or B-Raf mutations in any of the cell lines. Homogeneously, an activating H-RAS mutation in codon 61 was identified in MOC1/22 and the aggressive cell lines MOC2/7/10 bore an activating K-RAS mutation in codon 61 (Supplementary Fig. S4A). Evaluation of activated RAS identified that 5/6 cell lines had activated total RAS, and more specifically, the aggressive lines MOC2/7/10 had an approximate 2-fold increase in activated K-RAS compared with the indolent cell lines MOC1/22 (Supplementary Fig. S4B). Interestingly, the MOC23 cell line that formed tumors only in RAG2−/− immunodeficient mice had no RAS pathway mutations. This analysis of the MOC cell lines identified an oncogenic pathway that is upstream of ERK1/2 phosphorylation and potentially plays a primary role in the development and aggressiveness of mouse DMBA-induced OSCC.

**Phosphorylated ERK1/2 regulates OSCC migration and invasion**

The contribution of ERK1/2 in tumor cell migration and invasion in OSCC has received limited attention, and, therefore, we examined the effect of U0126, a selective inhibitor of the MEK1/2 kinases, in our system. Given the toxicity of U0126 (10 μmol/L) beyond 48 hours, we assessed the effect of U0126 on ERK1/2 phosphorylation, cellular proliferation, and migration after 24 hours. We found no effect on proliferation despite significant inhibition of ERK1/2 phosphorylation (Supplementary Fig. S5A and data not shown). Interestingly, the more aggressive MOC2 had nearly 4-fold higher basal capacity to migrate in Transwell assays compared with MOC1 (Fig. 2C and D), and this was proportionally inhibited to a higher extent by U0126 (93% for MOC2 vs. 51% for MOC1, significant interaction effect -Line×vehicle F(1,18) = 52.824, P < 0.001), suggesting a greater dependence on ERK1/2 for MOC2. Concordantly, U0126 treatment dramatically inhibited migratory ability of MOC2 in a scratch assay, whereas MOC1 was less affected (data not shown). Finally, assessment of invasion across a Matrigel matrix showed MOC2 had a greater ability to invade and was more attenuated upon U0126 treatment compared with MOC1 (Supplementary Fig. S5C and D). Thus, ERK1/2 activity is directly associated with migration and invasion in vitro.

**CD44 expression is correlated with increased ERK1/2 activation**

We next investigated the downstream targets of ERK1/2 that could be driving OSCC migration and invasion. Others have shown that ERK1/2 is associated with EMT, which itself is associated with CD44 expression (13, 30). Therefore, we assessed the expression of CD44 on the MOC lines and found increased cell surface CD44 on the aggressive MOC2/7/10 cell lines (MFI = 51, 42, and 76, respectively) compared with indolent MOC1/22 and 23 cell lines (MFI = 13, 9, 87, and 18, 6, Fig. 3A and B). This correlation was also detected in MOC1 and MOC2 cell lines growing in vivo (Fig. 4A, Supplementary Fig. S8). Thus, there is an association between tumor cell aggressiveness, ERK1/2 phosphorylation, and CD44 expression in our cell lines.

**CD44 is critical for MOC migration and in vivo growth**

We next addressed a causal connection between ERK1/2 activity, CD44, and migration/invasion. Three pan-CD44 shRNAs that target the CD44 coding region and a control scramble shRNA were used to create knockdown cell lines of MOC10 (CD44 knockdown of 70%, 88%, and 42%, Fig. 3C) and MOC2 (CD44 knockdown of 63%, 92%, and 29%, Supplementary Fig. S6A). Although the cells with CD44 knockdown morphologically seemed to be more clustered in culture (not shown), there was no effect on cell viability (Supplementary Fig. S6B) or alteration in ERK1/2 phosphorylation (Supplementary Fig. S6C). In contrast, reduced CD44 expression drastically inhibited in vitro cellular migration and invasion (Fig. 3D, Supplementary Fig. S6D, and data not shown) and in vivo growth and metastasis (Fig. 3E, Supplementary Fig. S6E and F and data not shown). Specifically, when assessed 50 days posttumor transplant, MOC10 scramble shRNA cells formed 20-mm tumors in 5/5 mice, with 4/5 displaying LN metastases, whereas MOC10 with silenced CD44 formed smaller (2–7 mm) tumors in fewer (6/10) mice, with no evidence of metastasis. Interestingly, when examined at later time points, some CD44...
knockdown tumors did contain metastatic LN deposits, which are likely escape mutants that had recovered CD44 expression (Supplementary Fig. S7). Similar results were found at a higher dose of tumor cells (Supplementary Fig. S6F), confirming that MOC cell CD44 plays a pivotal role in local aggressiveness and tumor growth in vitro and in vivo.

ERK1/2 regulates CD44 expression

Having established that CD44 contributes to growth in vivo and its surface levels correlated with phospho-ERK1/2 in vitro, we next tested whether this correlation was maintained in vivo. Transplanted MOC1 and MOC2 tumors were analyzed by immunofluorescence, which revealed that intracellular phospho-ERK1/2 staining coincided with cell surface CD44 for MOC2 (Fig. 4A, Supplementary Fig. S8). Although low levels of CD44 cell surface staining were detectable for MOC1, minimal phospho-ERK1/2 staining was detected (Fig. 4A).

We next employed loss- and gain-of-function approaches to assess whether ERK1/2 regulated CD44 expression. Blockade of ERK1/2 activation by U0126 consistently decreased CD44 surface expression compared with cells treated with vehicle control (Fig. 4B, Supplementary Fig. S9A and B). This effect of U0126 on CD44 expression was specific as cell surface CD24 was unchanged after 48 hours of U0126 treatment (Fig. 4B).

We then transduced the low phospho-ERK1/2 MOC1 cell line with a tamoxifen-inducible constitutively active MEK1/RAF construct (25). Tamoxifen treatment of these cells induced a 4-fold increase in cell surface CD44 compared with vehicle alone; however, tamoxifen treatment of parental MOC1 revealed no change in cell surface CD44 (Fig. 4C and
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Supplementary Fig. S9C). Thus, ERK1/2 activity is strongly linked to cell surface CD44 expression.

To address the mechanism of ERK1/2 regulation of CD44, MOC cell lines were assessed for differences in alternative splicing of CD44 and no isoform differences were found (data not shown). We next investigated whether the ERK1/2 pathway regulated CD44 gene expression, which has not been studied extensively but was suggested 18 years ago when Ponta and colleagues showed that c-H-RAS transcriptionally induced CD44 message (31). We found, using luciferase reporter constructs attached to basal (-97/+105) and full-length (-1,262/+105) sequences of the CD44 promoter (24), that cell surface CD44 expression correlated with luciferase activity as MOC2 and MOC10 had more (3- to 4-fold) full-length CD44 promoter–driven cell lines (31). We found, using luciferase reporter constructs attached to basal (-97/+105) and full-length (-1,262/+105) sequences of the CD44 promoter (24), that cell surface CD44 expression correlated with luciferase activity as MOC2 and MOC10 had more (3- to 4-fold) full-length CD44 promoter–driven luciferase activity compared with MOC1 (Fig. 4D). Importantly, this activity was reduced by mutating the AP-1 transcription factor–binding site (-1,262/+105 AP-1M; Fig. 4D) or by addition of U0126 (Fig. 4E). Finally, when MOCI cells were cotransfected with the full-length reporter and a tamoxifen-inducible MEK1/R4F construct, treatment with tamoxifen, but not vehicle, induced CD44 promoter activity (Fig. 4F). Together, these data support a model wherein an oncogenic RAS/MEK1/2/ERK1/2 and AP-1 cascade triggers transcription and surface expression of CD44 in MOC cells contributing to enhanced tumor aggressiveness.

ERK1/2 and CD44 in human OSCC

Having established the importance of the ERK1/2 and CD44 pathway in the aggressiveness of this murine model, we next extended these findings to human OSCC. Analysis of human OSCC cell lines revealed a spectrum of ERK1/2 phosphorylation levels. Of the cell lines analyzed, UM-SCC-1 had low levels and, UPCI:SCC029B, UPCI:SCC068, and PCI-13 had high levels of phospho-ERK1/2 (Fig. 5A). Interestingly, the primary tumor origin of these cell lines paralleled the MOC cell lines—cell lines with high p-ERK1/2 were derived from advanced tumors (all with nodal metastases), whereas the UM-SCC-1 cell line arose from a T2N0 primary tumor (32, 33). Similar to the MOC lines, fluorescence-activated cell sorting (FACS) analysis of CD44 showed a strict correlation with ERK1/2 phosphorylation, with low levels in UM-SCC-1 and high levels in UPCI:SCC029B, UPCI:SCC068, and PCI-13 (Fig. 5B). Inhibition of ERK1/2 activity by U0126 led to a modest decrease in cell surface CD44 expression (Fig. 5C and Supplementary Fig. S9D). In this particular model, it is possible that ERK1/2 activation is not the only driver of CD44 surface expression, and thus MEK inhibition may not be sufficient in all contexts to decrease CD44 levels. Finally, analysis of freshly resected primary human OSCCs by intracellular phospho-flow cytometry showed that the CD44high tumor cells had approximately 2- to 4-fold higher levels of phospho-ERK1/2 compared with the CD44low tumor cells (Fig. 5D and E and Supplementary Fig. S10). Moreover, transplantation of sorted CD44high and CD44low primary human OSCC cells into nonobese diabetic/severe combined immunodeficient gamma mice replicated published findings showing that CD44high tumor cells engraft better (data not shown; ref. 12). Thus, findings in the MOC lines parallel human OSCC.
Discussion

Herein, we describe a panel of transplantable syngeneic C57BL/6 OSCC cell lines that parallel the human disease in their (i) chemical carcinogenesis based mechanism of formation, (ii) intrinsic signaling aberrations, and (iii) histopathology and in vivo biology including lymphatic metastasis. Salley first described the use of DMBA as an oral carcinogen in the hamster cheek (34), but Crowe and colleagues only recently adapted this protocol to mice (26), which we then used to generate primary OSCCs and the cell lines. Using these resources, we identified that ERK1/2 activation and CD44 expression are linked, parallel findings in human OSCC and showed that ERK1/2 transcriptionally targets CD44 to contribute to an aggressive phenotype. These data define one mechanism whereby ERK1/2 mediates tumor aggressiveness and highlight the possibility of therapeutically targeting ERK1/2 in primary human OSCC.

The development of this murine model of OSCC represents a significant advance in the available preclinical tools for studying this disease. Currently used reagents include genetically engineered mouse models, such as those with oral mucosa specific inactivation of SMAD4, carcinogen-induced primary OSCC, and human tumor xenografts in immunodeficient mice (26, 35–37). Of these, the latter remain the most widely used by OSCC investigators. Xenograft models, although recapitulating the intrinsic signaling of the human disease, are not appropriate for in vivo studies of host–tumor interactions because they must be grown in immunocompromised mice. Transplantable syngeneic models for head and neck cancer have been described using oral keratinocytes, either transformed with 4-nitroquinolone or by expression of HPV E6/E7/H-RAS, but neither of these have been generally used and do not display fidelity with human OSCC in terms of lymphatic metastases (38, 39). Others have used the C3H/HeJ-derived SCCVII cell line, which is of cutaneous origin, as a surrogate for OSCC (40). Therefore, the panel of cell lines that we have generated helps fill the void of preclinical OSCC models by providing a transplantable, orally derived tumor system that can be grown in the universally used, immunocompetent C57BL/6 mice. Furthermore, and of paramount importance, this tumor system faithfully recapitulates several aspects of the human disease, including lymphatic metastasis.

We identified that the indolent MOC1/22 had an H-RAS, mutation whereas the more aggressive MOC2/7/10 cell lines had K-RAS mutations with 2-fold higher active K-RAS. This association between specific mutant RAS isoforms and tumor aggressiveness was similar to the finding of Chodosh and colleagues, who identified that in a c-MYC- or Wnt1-driven mouse model of breast cancer, tumors bearing K-RAS mutations had decreased oncogene dependence and increased ERK1/2 activation compared with ones with H-RAS mutations (41). K-RAS may induce additional effector pathways enhancing ERK1/2 phosphorylation as illustrated by studies of cell line resistance to the MEK inhibitor AZD6244 (42). Although mutant RAS and B-Raf oncogenic activators have been documented in human melanomas, pancreas, colon, and other cancers, the frequency of these mutations is far less in OSCC (43–45). In particular, RAS mutations (both in H- and K-RAS) have been reported at low frequencies in Western populations with OSCC. Interestingly, in India, where a common risk factor is the use of betel nut chew, the rate of RAS mutations in OSCC is as high as 35% to 50%, which may reflect an underlying genetic susceptibility or sensitivity to specific carcinogens (44–46).

In addition to the mutant RAS differences among the tumor cell lines, the major intracellular signaling alteration we identified was the degree of ERK1/2 phosphorylation. The slow growing MOC1/22 cell lines had markedly less ERK1/2 activation compared with the MOC2/7/10 lines. Activation of RAS/MEK1/2/ERK1/2 is commonly seen in malignancies and drives tumor cell proliferation, migration, and invasion by inducing antiapoptotic and proliferative pathways (47). ERK1/2 can also promote metastasis by inducing Slug, Snail, and EMT (30, 48). Recently, Blenis and colleagues showed that ERK2, but not ERK1, activated a Fra-1–mediated induction of ZEB1/2 to induce EMT in breast cancer (49). Extending these data, the connection we highlighted between ERK1/2 and CD44 in mouse and human OSCC cell lines and primary human tumors suggests a generalized connection between these molecules and is consistent with the description by Weinberg and colleagues of parallels between EMT and CSCs (13). Interestingly, Basu and colleagues recently identified that tumor heterogeneity, likely because of EMT, establishes chemotherapy resistance in a mesenchymal-like subset of cells and that drugs that target this population are ideal candidates for future therapeutics (50). Although MEK inhibitors have shown significant associated toxicities in clinical trials, including blurred vision and neurotoxicity, newer compounds are being developed that may have reduced effects in critical organs and may eventually contribute to oral cancer management (47). We speculate that ERK1/2 can, therefore, be targeted to prevent not only metastasis but also to treat chemoresistant CSCs that may express high levels of CD44 and ERK1/2 activity.

We found a key role for CD44 as a downstream effector of ERK1/2-mediated tumor aggressiveness. shRNA-mediated reduction of CD44 led to delayed tumor growth and the emergence of escape variants with parental levels of CD44, similar to the findings of Weinberg and colleagues (31). However, because these tumors recovered CD44 expression, we cannot make any definitive conclusion about the role of CD44 on metastasis with the knockdown approach. Thus, our data highlights the role of ERK1/2 and its downstream mediator CD44 in promoting OSCC aggressiveness using a new syngeneic model of transplantable OSCC. Our current work is focused on further mechanistic dissection of this link and therapeutically targeting both ERK1/2 and CD44 to alter the biology and outcomes of OSCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ERK1/2 Regulation of CD44 Modulates Oral Cancer Aggressiveness

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