EMP2 Is a Novel Regulator of Stemness in Breast Cancer Cells

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ABSTRACT

Little is known about the role of epithelial membrane protein-2 (EMP2) in breast cancer development or progression. In this study, we tested the hypothesis that EMP2 may regulate the formation or self-renewal of breast cancer stem cells (BCSC) in the tumor microenvironment. In silico analysis of gene expression data demonstrated a correlation of EMP2 expression with known metastasis-related genes and markers of cancer stem cells (CSC) including aldehyde dehydrogenase (ALDH). In breast cancer cell lines, EMP2 overexpression increased and EMP2 knockdown decreased the proportion of stem-like cells as assessed by the expression of the CSC markers CD44+/CD24−, ALDH activity, or by tumor sphere formation. In vivo, upregulation of EMP2 promoted tumor growth, whereas knockdown reduced the ALDH+ CSC population as well as retarded tumor growth. Mechanistically, EMP2 functionally regulated the response to hypoxia through the upregulation of HIF-1α, a transcription factor previously shown to regulate the self-renewal of ALDH+ CSCs. Furthermore, in syngeneic mouse models and primary human tumor xenografts, mAbs directed against EMP2 effectively targeted CSCs, reducing the ALDH+ population and blocking their tumor-initiating capacity when implanted into secondary untreated mice. Collectively, our results show that EMP2 increases the proportion of tumor-initiating cells, providing a rationale for the continued development of EMP2-targeting agents.

Introduction

Breast cancer is a leading cause of death among women, with over 90% of these deaths due to the development of metastasis (1, 2). Despite considerable progress in basic research leading to an increased understanding of breast cancer biology and advances in the treatment of early stage disease, metastatic breast cancer remains largely incurable (3). Substantial evidence suggests that one source of treatment resistance is the existence of cells that display “stem-like” properties. These cancer stem cells (CSC) are endowed with intrinsic properties that contribute to treatment resistance including increased DNA repair, expression of drug transporter proteins, antioxidant defenses, and slow cell-cycle kinetics (4, 5). In addition, their phenotypic plasticity facilitates their epigenetic reprogramming, rendering them resistant to multiple treatments including molecularly targeted agents (6).

A number of cell surface markers including CD44+/CD24− and enzymatic activities including aldehyde dehydrogenase (ALDH) are associated with cells that display increased tumor-initiating capacity in mouse xenograft models (7, 8). These cells also display increased invasive capacity and may play a fundamental role in mediating tumor metastasis (8, 9). Furthermore, the expression of these CSC markers correlates with poor prognosis in patients across a spectrum of tumor types including breast cancer (10–12). These data highlight the importance of developing therapeutic strategies capable of successfully targeting the CSC population.

One strategy to accomplish this is to identify and target CSC regulatory proteins. Epithelial membrane protein-2 (EMP2) is a tetraspan protein, which has been reported to be overexpressed in a number of cancers including its expression in 63% of invasive breast cancers where EMP2 levels correlate with disease progression (13). In addition, its expression has been reported in breast metastases as well as in circulating tumor cells (13–15). However, little is known about its role in disease etiology.

In this study, the role of EMP2 in breast cancer progression and in the regulation of breast cancer stem cells (BCSC) was investigated using cell lines and xenograft models. The clinical relevance of these studies was supported by in silico analysis of TCGA datasets, demonstrating a correlation of EMP2 expression with markers of metastasis in breast cancers across a spectrum of breast cancer subtypes. Using gene knockdown, we validated EMP2 as a CSC regulatory target. We demonstrate that a mAb directed against EMP2 reduces CSCs in vitro and blocks tumor-initiating capacity and tumor growth in mouse xenograft models. Collectively, these studies support the
continued development of anti-EMP2 therapeutics to target the CSC population, which mediates tumor metastasis and treatment resistance.

**Materials and Methods**

**Cell culture**

The human breast cancer cell lines MDA-MB-231, MDA-MB-468, HS578T, BT474, BT-20, MDA-MB-157, MCF7, ZR-75-30, and SKBR-3 (ATCC) were cultured in DMEM with 10% FCS (HyClone Laboratories), 1% sodium pyruvate, 1% penicillin/streptomycin, and 1% L-glutamine (Life Technologies) at 37°C in a humidified 5% CO2. HCC1806, HCC202, MDA-MB-453, HCC1419 (all ATCC), and JIMT-1 (German Tissue Repository; DSMZ) were maintained in RPMI1640 supplemented with 10% heat-inactivated FBS, 1% penicillin G-streptomycin-fungizone solution (PSF, Irvine Scientific), 1% MEM (University of Michigan, Ann Arbor, MI) cells were grown in DMEM/F12 medium supplemented with 10% heat-inactivated FBS, 1% PSF, 0.5 mM glutamine, 1% penicillin/streptomycin, 1% sodium pyruvate, 1% MEM and UACC-732 cells were maintained in MEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 1% PSF, 1% MEM nonessential amino acids, and 1 μmol/L sodium pyruvate. SUM190 (University of Michigan, Ann Arbor, MI) cells were grown in DMEM/F12 medium supplemented with 10% heat-inactivated FBS, 1% PSF, 10 μg/mL insulin, and 1 μg/mL hydrocortisone. MCF10A and MCF12A cells (kind gift from Jaydutt Vadgama, Charles Drew University) were grown in Dulbecco’s Modified Eagle’s/F12 medium (DMEM/F12, 1:1) containing 5% equine serum (ES), 20 ng/mL EGF, 0.5 μg/mL hydrocortisone, 0.1 μg/mL cholaer toxin, and 10 μg/mL insulin. Cells were passaged weekly and tested periodically for Mycoplasma contamination using MycoAlert Kit (Lonza). SUM149PT cells were maintained in Ham’s F12K media supplemented with 5% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone, and 4 μg/mL gentamicin in addition to the supplements listed above. Finally, 4T1 cells, a spontaneous mammary tumor syngeneic in BALB/c mice, were obtained from ATCC. 4T1 cells were maintained in RPMI medium supplemented as above. Both 4T1 and MDA-MB-231 cells were stably infected with firefly luciferase (FLUC) as described previously (16). Cell lines were used within 3 months after resuscitation of frozen aliquots and were authenticated based on viability, recovery, growth, morphology, and isoenzymology by the supplier. Modulation of EMP2 expression in cell lines is described in the Supplementary Methods.

**Production of the anti-EMP2 mAb**

Anti-EMP2 mAb is currently produced in bulk by Lake Pharma on a contractual basis according to their standard practices. The variable sequences used to generate the mAb are provided below:

**HC-hmAb:** QVQLVQSLGSQMVGVQPSLRLSLCCLASSEGGGSYALMHWVRQAPGKGEGWAVLYGNGSRYADVGKVRFTISRDNSKNTLYQMSNLRAEDTAVYLCARDRGRKRSGAGIDYWGQGTLYVSS

**LC-hKappa:** DIQMTQSPSLASSVGHRVTITCQAQSIDNSYLNWWYQQKPGKFLIYAAASLQSGVPSRFSGSSGTDFTLTISSLQPEDFTTTYYCTQLDYNGWTFGQGTKV-DIK

The affinity of the antibody is verified against a specific and scrambled EMP2 peptide using ForteBio Octet with an affinity between 5 and 8 nM is considered acceptable.

**TCGA data**

The expression of EMP2 in breast cancer was obtained through the CBioPortal for Cancer Genomics (http://cbioportal.org; ref. 17). 1105 provisional cases of invasive breast cancer were evaluated for EMP2 expression (RNA Seq V2 BSEM; Z-score threshold ± 1.1). A minimum criteria of at least 20 (out of 814) cases was set. From the dataset, the genes that coexpressed with EMP2 were selected and used to generate a ranked list (from high to low correlation). This ranked list was then analyzed using gene set enrichment analysis (GSEA; http://software.broadinstitute.org/gsea/index.jsp), which identified 61 gene sets that had an FDR q-val < 0.5.

**IHC**

**Patient samples**

Approval from the Institutional Review Board at UCLA (Los Angeles, CA) was obtained before initiating the study. EMP2 and ALDH1 expression was measured in 62 patients with breast cancer. The patients represent the population of women who had undergone breast surgery at King/Drew Medical Center (Willowbrook, CA) from 1999 to 2005, and profiles as 57% African American, 37% Latina, 1% Asian, and 4% Caucasian. Samples were stained as described in the Supplementary Methods section.

**Treatment and analysis using mouse xenograft model**

Animal studies were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH (Bethesda, MD). The Animal Research Committee at the University of California, Los Angeles or the University of Michigan approved all procedures. All efforts were made to minimize animal suffering. The Supplementary Methods section describes the models created.

Information about SDS-PAGE/Western blot analysis, RNA sequencing, flow cytometry, tumorspheres, and statistical analyses are described in the Supplementary Methods.

**Results**

**EMP2 is expressed in human breast cancers and is associated with metastasis**

In a previous study, EMP2 protein was shown to be upregulated in all subsets of breast cancer (13), but little is known about its role in the etiology or clinical course of this disease. To address this, EMP2 expression was queried using data generated by the TCGA Research Network: http://cancergenome.nih.gov/. Concordant with previous publications on EMP2 protein expression, EMP2 mRNA expression was significantly increased in women with breast cancer, with 28.4% of ductal carcinomas and 20.3% of lobular breast cancers showing increased expression (Supplementary Fig. S1A). Mixed ductal and lobular carcinomas also showed increased EMP2 mRNA expression in 29.6% of patients. To next elucidate gene expression changes associated with EMP2 mRNA upregulation, GSEA was performed on a ranked list of genes coexpressed with EMP2. EMP2 was coexpressed with genes enriched in all breast cancer classes, with hypoxia, and genes sets associated with breast cancer metastasis and disease relapse (Supplementary Fig. S1B; Supplementary Table S1).

**EMP2 affects tumor growth in mouse xenografts**

Initially, the expression of EMP2 was analyzed in cell lines representing luminal, basal, mesenchymal, and HER2 subtypes of disease as well as in two normal mammary cell lines (MCF10A and MCF12A;
EMP2 expression promotes tumor growth. **A,** A panel of normal mammary cell lines and breast cancer cell lines (MCF10A and MCF12A) representing the major molecular subtypes were tested for EMP2 expression with β-actin used as a loading control. **B,** To determine the role of EMP2 in tumorigenesis, BT474 breast cancer cells with high EMP2, endogenous, or low levels (downregulation through sh KD #1) were injected in Balb/c nude mice. Quantitation of EMP2 expression in the different cell lines is depicted on the left. The growth of the cells over 40 days is shown in the middle, with final tumor volumes illustrated on the right. *n* = 4. Significance was determined using two-way ANOVA, *P* < 0.0001, and results from Bonferroni multiple comparisons test shown in the figure. **C,** MDA-MB-231 cells with modified EMP2 levels as above were injected into Balb/c nude mice and measured over 62 days. Significance using a two-way ANOVA, *P* = 0.0004. Bonferroni multiple comparisons was used tested post hoc with results shown in the figure. *n* = 5. Right, final tumor volumes are highlighted. **D,** EMP2 expression in SUM149 cells were modified to overexpress or reduce EMP2 levels (sh KD#2) compared with a vector control. Cells were injected into Balb/c nude mice, and tumor load monitored over 45 days. Significance using a two-way ANOVA, *P* = 0.009. Post hoc analysis using Bonferroni multiple comparisons test is shown in the figure. *n* = 4. Right, graph illustrates final tumor weights. **E,** Transcriptomic data showing differential expression in SUM149 cell line infected with a vector control or shRNA lentiviral vector (sh KD#2). TMM normalized read counts per million were utilized to generate a heatmap based on differential expression of CD44, CD24, and various ALDH isoforms. EMP2 significantly altered the expression of multiple ALDH isoforms, and it was associated with increased CD24 and reduced CD44. *, *P* < 0.05.
EMP2 Enhances Cancer Stem Cell Formation

Supplementary Table S2; refs. 18, 19). As demonstrated in Fig. 1A, low levels of EMP2 were detectable in both MCF10A and MCF12A. However, significantly higher levels of the protein were present in all breast cancer cell lines with the exception of HS578T, a breast carcinoma cell line. To investigate its functional role within these cells, we overexpressed or knocked down EMP2, utilizing specific shRNAs in cell lines representing major breast cancer subtypes. Overexpression of EMP2 accelerated and knockdown significantly inhibited the growth of both hormone receptor-positive and triple-negative breast cancer (TNBC) cells lines represented by BT474 and MDA-MB-231 (Fig. 1B and C). In the case of SUM149, whereas knockdown of EMP2 significantly inhibited tumor growth compared with the vector control, less pronounced changes were observed upon overexpression (Fig. 1D). As all three cell lines exhibited reduced tumor load concomitant to lower EMP2 levels, the effect of EMP2 knockdown was further investigated.

EMP2 knockdown affects expression of genes associated with cancer “stemness”

To elucidate pathways regulated by EMP2, we determined the effect of EMP2 knockdown on gene expression in SUM149 cells via RNA sequencing (RNA-seq). Comparison of the vector control versus shRNA knockdown revealed 332 genes with differential expression between the two groups. GSEA revealed a significant enrichment, relative to EMP2 levels, for the response to hypoxia, similar to the enrichment seen using TCGA data as above (Supplementary Table S3).

Substantial evidence now exists that many cancers including breast cancer are hierarchically organized and contain subpopulations of cells that display stem cell properties. These cells play putative roles in treatment resistance and metastasis (10, 20). Several markers including CD44+/CD24− and ALDH activity have been shown to enrich for tumor cells displaying “stem-like” properties. As hypoxia is a known regulator of CSCs (5, 21), we interrogated the RNA-seq data to elucidate the effect of EMP2 knockdown on CSC marker expression. As shown in Fig. 1E, EMP2 knockdown increased CD24 expression while decreasing expression of CD44 as well as a number of ALDH1, the effects of EMP2 knockdown on ALDH activity were significantly reduced ALDH activity as assessed by the ALDEFLUOR assay correlated with ALDH1 protein expression, and similarly, levels of EMP2 were significantly overlapped with 65% to 99% of CD44+/CD24− cells in all cell lines (Supplementary Fig. S1C), and its expression was enriched in cells displaying an ALDHhigh phenotype. ALDHhigh and ALDHlow cells were sorted and analyzed for ALDH1 and EMP2 expression using Western blot analysis (Supplementary Fig. S1D). In all three cell lines tested, ALDH activity as assessed by the ALDEFLUOR assay correlated with ALDH1 protein expression, and similarly, levels of EMP2 were higher in ALDHlow compared with ALDHhigh populations (Fig. 2C). To validate this finding, the overlap between ALDH activity and EMP2 levels was determined using flow cytometry using diethylaminobenzaldehyde or DEAB, a specific inhibitor of ALDH, as a negative control. All ALDHhigh cells showed high expression of EMP2, with results from representative cell lines provided (Supplementary Fig. S1E).

Correlation of HIF-1α, ALDH, and EMP2 expression

To further our understanding of the relationship between EMP2 and ALDH1, the effects of EMP2 knockdown on ALDH activity were analyzed using DEAB as a negative control. In these cells, knockdown of EMP2 significantly reduced ALDH activity as assessed by the ALDEFLUOR assay (Fig. 3A). To verify the dependence of ALDH1 expression on EMP2, cells were next transiently transfected with an EMP2 siRNA or scrambled control. Similar to effects observed with the shRNA knockdown, transient knockdown of EMP2 using a pooled set of four siRNA significantly reduced ALDH1 expression (Fig. 3B). These results validate the correlation of EMP2 expression with ALDH1 expression in vitro.

Previous studies have shown links between EMP2 and hypoxia (28) as well as hypoxia and the formation of BSCCs (29, 30). To determine whether exposure to hypoxia alters EMP2 and ALDH1 expression, two representative cell lines were placed in a hypoxic chamber for 24 hours and EMP2 and ALDH1 levels analyzed by Western blot analysis. In response to hypoxia exposure, expression of HIF-1α, ALDH1, and EMP2 were all significantly increased (Fig. 3C). To extend upon this observation, cells with varying levels of EMP2 were placed in hypoxic conditions for 24 hours. In all 4 cell lines, HIF-1α expression directly correlated with EMP2 levels (Fig. 3D; Supplementary Fig. S1F), suggesting that EMP2 helps regulate the hypoxic response of breast cancer cells.

Clinical correlation of ALDH and EMP2 expression

To investigate the clinical relevance of these findings, we utilized IHC to examine the relationship between EMP2 and ALDH1 expression in breast cancer biopsies. The patient cohort consisted of 24 women with TNBC, 4 women with HER2+, hormone receptor
negative (HR- / C0) disease, 23 women with luminal A (ER+ and/or PR+/ HER2-/ C0) disease, and 11 women with luminal B (ER+ and/or PR+/ HER2+) disease. Although all women analyzed presented with invasive ductal carcinoma (IDC), foci of ductal carcinoma in situ (DCIS) were also observed.

High levels of EMP2 were observed in both DCIS and IDC spots independent of hormone status compared with histologically "normal" lobules adjacent to the tumor (Fig. 4A; Table 1). No statistical differences were observed in the mean expression of EMP2 in DCIS foci and IDC (1.84 ± 0.22 and 1.86 ± 0.08, respectively), and both were

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Figure 2.
EMP2 expression promotes tumorsphere formation and markers associated with stemness. A, The effects of EMP2 expression on mammosphere formation was evaluated in MDA-MB-231 and BT474. EMP2 levels directly correlated with increased mammosphere formation. n = 4; significance was determined using Student t test. B, Breast cancer cells express varying levels of CD44+/CD24- or ALDH activity as determined by the ALDEFLUOR assay. Left, the percentage of CD44+/CD24- cells observed in a panel of breast cancer cell lines as determined by flow cytometry. Middle, percentage of EMP2+ cells was determined in cells gated on CD44+/CD24- using flow cytometry. Right, assessment of ALDH activity in a panel of breast cancer cell lines as measured by the ALDEFLUOR assay. n = 3 with results expressed as the mean ± SEM. C, High levels of ALDH1 correlated with high levels of EMP2 expression. Cells were sorted into ALDHhigh or ALDHlow as determined using the ALDEFLUOR assay and subsequently analyzed for EMP2 and ALDH1 expression by Western blot analysis. Results were normalized to β-actin levels and tabulated as the mean ± SEM, with significance determined using Student t test. n = 3.
Figure 3.
ALDH activity changes relative to EMP2 expression. A, As a functional readout of ALDH activity, breast cancer cells with endogenous or shRNA knockdown of EMP2 were tested using the ALDEFLUOR assay. A specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), was used as a negative control. Higher EMP2 levels correlated with an increase in the number of ALDH-high cells.
B, To independently confirm the regulation of ALDH1 by EMP2, cells were transiently transfected with an EMP2 or scrambled siRNA. Knockdown of EMP2 levels significantly reduced ALDH1 expression in two representative cell lines. 
C, Hypoxia increases ALDH1 and EMP2 levels. MDA-MB-468 and SKBR3 were plated under both normoxic and 0.1% O2 hypoxic conditions. After 24 hours, ALDH1 and EMP2 levels were measured relative to β-actin. A representative Western blot is shown on the left, and results tabulated from three independent experiments shown on the right. Data are expressed as the mean ± SEM. D, SKBR3, MDA-MB-468, BT474, and MDA-MB-231 cells were placed in 0.1% O2 hypoxic chamber. HIF-1α expression was monitored in response to changes in EMP2. All experiments were repeated 3 times, with results expressed as the mean ± SEM. P values show significant differences between groups using Student t test.
Figure 4.
ALDH1 and EMP2 are coexpressed. A, EMP2 and ALDH1 expression was measured in a tissue microarray from 62 women with invasive ductal carcinoma. Relative expression measures both the percentage of positive cells and the intensity of their staining, with a maximum score of 3. EMP2 and ALDH1 expression was significantly upregulated in cancer compared with adjacent normal tissue (P = 0.002 and P = 0.0002, respectively; Kruskal-Wallis). Within the same patient, no significant differences were observed in DCIS relative to IDC for both ALDH1 and EMP2. B, Correlation of EMP2 and ALDH1 in patient samples. Although all ALDH1-positive tumors were EMP2 positive, the relative expression of EMP2 did not correlate with that of ALDH1 in the primary tumor (n = 47). Middle, a significant correlation between the percentage of ALDH1 and EMP2 cells occurred within lymph node metastasis (P = 0.04; n = 19). Right, this correlation was also significant when expression intensity was included (P = 0.05). In all graphs, the intensity of color (darkness) is proportional to the number of samples at a given location. C, ALDH1 expression (brown) was measured in MDA-MB-231, BT474, and SUM149 xenografts that overexpress EMP2, express a vector control, or express EMP2 shRNA knockdown tumors. Representative images are shown at a × 400 magnification, with some ALDH1-positive cells highlighted with arrows. Scale bar, 50 µmol/L. n = 4 with results shown as the mean ± SEM.
Table 1. Characterization of 62 patients with breast cancer stained for EMP2 or ALDH1.

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Patient overall histology comparisons

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Note: An adjusted histologic score of 0.4 was used to set the threshold for positivity based on staining in a positive and negative control tissue. EMP2 and ALDH1 are present in all breast cancer subtypes. Abbreviation: n, tumor core samples with adequate marker information.

Anti-EMP2 mAb reduces the proportion of BCSCs in vitro

As the above data suggested that downregulation of EMP2 reduced markers and activities associated with BCSCs, we next determined whether an anti-EMP2 mAb (13) could produce a similar effect. Consistent with previous reports, ALDH1 displayed a heterogeneous staining pattern within select patients with breast cancer, which was shared across molecular reports, ALDH1 displayed a heterogenous staining pattern within select patients with breast cancer, which was shared across molecular reports. EMP2 expression was often observed both within the parenchyma as well as within selective samples with increased ALDH1 positivity. However, this occurred in both representative metastatic lesions (Supplementary Fig. S2D) as well as in patients with IDC (Supplementary Fig. S2E), although notably, many IDC samples did not express detectable ALDH1 (Supplementary Fig. S3F). Together, these results suggest that EMP2, ALDH1 coexpressing cells may represent a subset of the EMP2<sup>+</sup> primary tumor with increased metastatic capacity. Further studies testing if EMP2 regulates metastatic potential will be required to test this hypothesis.

To extend this observation, the expression of EMP2 and ALDH1 was next examined in the xenograft models created from MDA-MB-231, BT474, and SUM149 described above. All three cell lines in vivo showed increased numbers of ALDH1-positive cells with increased EMP2 expression. In contrast, low to moderate levels of ALDH1 were observed in the vector control samples, while knockdown of EMP2 produced a statistically significant reduction in its levels (Fig. 4C). These results suggest that knockdown of EMP2 may sufficiently target this population of cells.

Table 2. Patient subgroup characteristics and core tissue sample comparisons.

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Note: An adjusted histologic score of 0.4 was used to set the threshold for positivity based on staining in a positive and negative control tissue.

Abbreviation: n, tumor core samples with adequate marker information.

*Tumor adjacent.

higher than the relative expression observed in normal breast tissue (P = 0.002, Kruskal–Wallis). Interestingly, in 68% of patients, adjacent “normal” tissue showed some low levels (0.02 ± 0.11) of EMP2 as well. Supplementary Figure S2A illustrates staining between normal, DCIS, and IDC spots. Although our results suggest that EMP2 may be expressed at preinvasive stages of breast cancer progression, it is also possible that the similar expression observed in DCIS and IDC may be attributable to “field effects” (31).

Sections were also stained for ALDH1. Consistent with previous reports, ALDH1 displayed a heterogeneous staining pattern within select patients with breast cancer, which was shared across molecular breast cancer subtypes (Table 1). Within the tumor, ALDH1 expression was often observed both within the parenchyma as well as within the tumor stroma (Supplementary Fig. S2B, ref. 32). Twenty-six of 47 patients (55.3%) expressed ALDH1 within the tumor parenchyma albeit at low levels, and its expression was higher in cancer compared with normal epidermis (P = 0.0002, Kruskal–Wallis; Fig. 4A). Similar to EMP2, no significant differences were observed in the relative expression of ALDH1 in DCIS and IDC from these patients (0.18 ± 0.08 and 0.23 ± 0.04, respectively). In normal breast tissue, minimal expression of ALDH1 was observed within epidermis (0.1 ± 0.01; Supplementary Fig. S2C).

Correlation of EMP2 and ALDH1 expression showed that all patients with ALDH1<sup>+</sup> cells were EMP2<sup>+</sup> as well. However, when the intensity of staining was taken into account, there was no direct correlation between the expression of EMP2 and ALDH1 within the primary tumor (Fig. 4B). This likely reflects the low overall staining observed with ALDH1 relative to EMP2. In addition to the primary tumor, the tissue array also included lymph nodes with metastatic disease from a subset of patients, allowing for the analysis of protein expression in these lesions. A significant correlation between EMP2 and ALDH1 occurred within lymph node metastases (Fig. 4B). The trend of EMP2 and ALDH1 positivity within lymph nodes was significant both based on percent positivity and intensity of expression. To confirm these results, a limited number (n = 10) of full section metastatic lesions or IDC samples were independently double stained for both markers. Similar to the results obtained above, all ALDH1-positive tumor cells (visualized as brown) coexpressed EMP2 as well (visualized as red). This occurred in both representative metastatic lesions (Supplementary Fig. S2D) as well as in patients with IDC (Supplementary Fig. S2E), although notably, many IDC samples did not express detectable ALDH1 (Supplementary Fig. S3F). Together, these results suggest that EMP2, ALDH1 coexpressing cells may represent a subset of the EMP2<sup>+</sup> primary tumor with increased metastatic capacity. Further studies testing if EMP2 regulates metastatic potential will be required to test this hypothesis.

To extend this observation, the expression of EMP2 and ALDH1 was next examined in the xenograft models created from MDA-MB-231, BT474, and SUM149 described above. All three cell lines in vivo showed increased numbers of ALDH1-positive cells with increased EMP2 expression. In contrast, low to moderate levels of ALDH1 were observed in the vector control samples, while knockdown of EMP2 produced a statistically significant reduction in its levels (Fig. 4C). These results suggest that knockdown of EMP2 may sufficiently target this population of cells.

Anti-EMP2 mAb reduces the proportion of BCSCs in vitro

As the above data suggested that downregulation of EMP2 reduced markers and activities associated with BCSCs, we next determined whether an anti-EMP2 mAb (13) could produce a similar effect. These results provide validation of the integrity and affinity of the anti-EMP2 mAb, respectively. To test the effects of the mAb, MDA-MB-468 cells were treated with control human mAbs or anti-EMP2 mAbs for 1 to 5 days, and EMP2 and ALDH1 levels relative to β-actin were quantitated. Relative to the control, anti-EMP2 mAbs significantly reduced EMP2 (P = 0.02, day 5) and ALDH1 (P = 0.0002, day 5) levels (Fig. 5A).

To corroborate these findings, changes in ALDH activity were next determined using the ALDEFLUOR assay. MDA-MB-468, SKBR3, and MDA-MB-231 cell lines were incubated with anti-EMP2 or control mAbs for 24 hours, and changes in ALDH activity measured relative to the DEAB inhibitor. In all three cell lines, anti-EMP2 mAbs significantly reduced ALDH activity by 50% to 80% compared with those treated with control mAbs (Fig. 5B).

We next tested the ability of the anti-EMP2 mAbs to reduce the self-renewal capability of CSCs using a tumorsphere assay. Initially, 5000 BT474 cells were treated with saline or 50 μg/mL control human or anti-EMP2 mAbs and cultured for 2 weeks under low adherence conditions. Anti-EMP2 mAbs significantly reduced tumorsphere forming efficiency compared with vehicle and mAb controls (Fig. 5C). To extend upon these observations, we tested the ability of the anti-EMP2 mAbs to reduce the self-renewal capability of CSCs using a single cell tumorsphere assay. One, 5, or 10 live cells were incubated with 10 μg/mL of control or anti-EMP2 mAb, and viable tumorspheres enumerated after two weeks (Table 2). Treatment with anti-EMP2 mAbs significantly inhibited tumorsphere formation in the multiple cell lines tested.

Several groups have shown that CSC subpopulations can arise during tumor progression (33). To study the emergence of CSC subpopulations during this process in vivo, we next tested the ability of the anti-EMP2 mAbs to target xenograft-derived cells. MDA-MB-231 and primary HCI-002 xenografts were created, and cells were isolated from harvested tumors and plated as above. In both
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A

<table>
<thead>
<tr>
<th>Ctrl mAb</th>
<th>EMP2 mAb</th>
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<tbody>
<tr>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>EMP2</td>
<td>-</td>
</tr>
<tr>
<td>ALDH1</td>
<td>-</td>
</tr>
<tr>
<td>β-Actin</td>
<td>-</td>
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B

SSC

C

Un-treated

D

MDA-MB-231

E

PBS

F

Reinjection

G

Reinjection

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cases, anti-EMP2 mAbs significantly inhibited tumoursphere formation compared with control mAbs (Fig. 5D).

### Anti-EMP2 mAb reduces tumor growth and tumor-initiating potential

Our results thus far suggested that anti-EMP2 mAbs may successfully reduce markers and features associated with BCSCs. To translate this observation, we utilized the models tested above to determine the effects of anti-EMP2 mAbs in vivo. Initially, primary HCl-002 tumor pieces were implanted into NOD/SCID animals, and when tumors reached approximately 50 to 75 mm³, treatment was initiated. Anti-EMP2 mAb significantly reduced tumor growth of the primary tumors compared with control mAbs (Fig. 5E, two-way ANOVA, \( P = 0.02 \)). We next determined the effects of treatment on ALDH1 and EMP2 expression using standard IHC (Supplementary Fig. S3C). A quantitative reduction in the percentage and intensity of EMP2 and ALDH1 expressions were observed post-anti-EMP2 mAb treatment compared with the control as scored by a pathologist (Y. Elshimali) masked to the treatment groups (\( P = 0.002 \) and \( P = 0.04 \), respectively; Fig. 5E, right).

To extend upon these observations, a second model was created using MDA-MB-231 cells. In two independent experiments, treatment with anti-EMP2 mAb significantly reduced tumor load compared with treatment with control human mAbs (Fig. 5F; Supplementary Fig. S3D and S3E). As the “gold standard” assay for CSCs is their ability to initiate tumors in secondary mice, two assays were performed on remnant cells of the tumor to determine whether anti-EMP2 mAbs were sufficient to prevent tumor cell spheres to form tumors. anti-EMP2 mAbs were probed by Western blot analysis for EMP2 or ALDH1 expression for up to 120 hours. Bottom, densitometric analysis of Western blots for expression of EMP2 or \(-\alpha\)-actin. Samples were normalized to the 24-hour controls.

### Table 2. Tumoursphere formation of breast cancer cells treated with Anti-EMP2 mAb.

<table>
<thead>
<tr>
<th># Spheres/wells</th>
<th>Treatment</th>
<th>1 cell/well</th>
<th>10 cells/well</th>
<th>50 cells/well</th>
<th>1/TIC frequency (95% CI)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT474</td>
<td>Control</td>
<td>49/128</td>
<td>113/128</td>
<td>126/128</td>
<td>1/7.62 cells (6.82-8.5)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Anti-EMP2 mAb</td>
<td>30/128</td>
<td>96/128</td>
<td>113/128</td>
<td>1/12.99 cells (11.69-14.4)</td>
<td>5.05e-15</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>31/120</td>
<td>109/120</td>
<td>144/144</td>
<td>1/3.31 cells (2.93-3.73)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Anti-EMP2 mAb</td>
<td>12/120</td>
<td>66/120</td>
<td>143/144</td>
<td>1/8.97 cells (8.02-10.03)</td>
<td>2.70e-32</td>
</tr>
<tr>
<td>SUM149</td>
<td>Control</td>
<td>17/1/384</td>
<td>ND</td>
<td>ND</td>
<td>1/1.98 cells (1.81-2.18)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Anti-EMP2 mAb</td>
<td>12/384</td>
<td>ND</td>
<td>ND</td>
<td>1/2.64 cells (2.38-2.93)</td>
<td>5.1e-05</td>
</tr>
</tbody>
</table>

Note: Either 1, 10, or 50 live cells were FACS sorted into 50 \( \mu \)L of enhanced serum-free media (± \( \mu \)g/mL anti-EMP2 mAb) per well in nontreated 384-well plates. Spheres were counted 2 weeks later. The frequency of tumoursphere-initiating cells was calculated using the online extreme limiting dilution analysis calculator (http://biostat.wisc.edu/software/elda/index.html). \( P \) values were calculated by testing for inequality in frequency between multiple groups.

### Figure 5.

Anti-EMP2 mAbs reduce markers associated with stemness. A, MDA-MB-468 cells were treated with \( 50 \mu \)g/mL of anti-EMP2 or control human mAbs, and cell lysates were probed by Western blot analysis for EMP2 or ALDH1 expression for up to 120 hours. Bottom, densitometric analysis of Western blots for expression of EMP2 or ALDH1 relative to \(-\alpha\)-actin. Samples were normalized to the 24-hour controls. \( n = 3 \). \( P < 0.05 \) by Student t test. B, MDA-MB-468, SKBR3, and MDA-MB-231 cells were treated with \( 50 \mu \)g/mL anti-EMP2 or control mAbs (anti-CD20) for 24 hours to monitor ALDH activity via the ALDEFLUOR assay. Diethylaminobenzaldehyde or DEAB, a specific inhibitor of ALDH, was used as a negative control. The graphs represent the average ± SEM of ALDH activity following treatment with control or anti-EMP2 mAbs from three independent experiments. C, Left, 5,000 BT474 cells were left untreated, treated with \( 50 \mu \)g/mL control human, or treated with anti-EMP2 mAbs and cultured for 2 weeks under low adherence conditions. Tumourspheres were enumerated, with representative examples shown. Below, quantitation of three independent experiments showing the average tumoursphere forming efficiency ± SEM, with significance determined using Student t test. Magnification = ×100. D, MDA-MB-231 or HCl-002 cells were isolated from xenografts created in nude or NOD/SCID animals, respectively. Isolated cells (5,000) were cultured in triplicate and treated as above with control human mAbs or anti-EMP2 mAbs. The graphs below represent the averaged results ± SEM from triplicate samples prepared from two independent experiments. Magnification = ×100. E, The triple negative primary tumourgraf HCl-002 was implanted into the mammary fat pad of NOD/SCID animals. Grouped and individual data are provided for animals treated with \( 10 \) mg/kg twice a week with either control mAbs or an anti-EMP2 mAb. \( n = 4 \). The arrow denotes when tumor treatment was initiated. Two-way ANOVA, \( P = 0.02 \). \( \dagger \) Significance by Bonferroni multiple comparisons test. Right, at day 71, tumors were harvested, fixed, and stained for ALDH1 or EMP2 expression using standard IHC. The graphs show the average IHC intensity of each protein, ± SEM, \( n = 4 \). Significance was determined using Student t test. F, A total of \( 1 \times 10^6 \) MDA-MB-231 cells were implanted into the mammary fat pad of Balb/c nude mice. When tumors averaged 80 to 100 mm³, they were grouped and treated with human control or anti-EMP2 mAbs at \( 10 \) mg/kg twice a week. \( n = 4 \). Two-way ANOVA, \( P = 0.001 \). \( \ddagger \) Significance by Bonferroni multiple comparisons test. Right, following treatment, tumors were dissociated, and some of the cells monitored for ALDH activity via the ALDEFLUOR assay. Results are expressed as the average ± SEM, \( n = 4 \). G, A single-cell suspension of the resected primary tumor initially treated with control or anti-EMP2 mAbs was reinjected into secondary animals at either 500, 5,000, or 50,000 cells. No additional treatments were given. In animals reinjected with 50,000 cells, tumor load was monitored and the mean ± SEM illustrated, \( n = 4 \). Two-way ANOVA, \( P = 0.001 \). \( \ddagger \) Significance by Bonferroni multiple comparisons test.
reinitiation. After tumor cell disassociation, cells were either analyzed for ALDH activity or reinserted into naïve animals to monitor secondary tumor formation. Treatment with anti-EMP2 mAb produced a marked reduction in ALDH activity as measured by the ALDEFLUOR assay compared with those treated with human control mAbs (Fig. 5F, right). To further assess the ability of remnant cells to initiate tumor formation, we injected serial limiting dilutions (50,000, 5,000, or 500 cells) into nude mice and monitored tumor formation without any further treatment. Figure 5G follows tumor reinitiation from cells injected with 50,000 cells, and results indicate that anti-EMP2 mAbs dampen the efficiency of tumor reinitiation. Similarly, at all three doses of cells, a significant inhibition in secondary tumor formation was observed after anti-EMP2 treatment compared with the control, suggesting that anti-EMP2 mAbs effectively target a significant portion of BCSCs within this tumor model (Supplementary Table S5).

Anti-EMP2 mAb reduces tumor metastasis

It has been suggested that CSC dictate the fate and behavior of the tumor (33). As in silico analysis suggested an enrichment of genes involved in metastasis with higher EMP2 levels, we determined whether anti-EMP2 mAbs could inhibit metastasis in an experimental mouse xenograft model. Luciferase-labeled MDA-MB-231 cells were injected into the left ventricle of Balb/c nude mice, and animals were treated twice weekly with 10 mg/kg control or anti-EMP2 mAbs. Tumor metastasis was monitored using bioluminescence imaging. As shown in Fig. 6A, anti-EMP2 treatment selectively produced a quantitative reduction in tumor metastasis.

Previous studies have shown that anti-human EMP2 mAbs cross react with murine EMP2 (13), and we thus hypothesized that these mAbs may reduce the metastatic burden in a 4T1 syngeneic model. Given that these animals have an intact immune system, they may also be more clinically relevant. 4T1 cells labeled with FLUC were injected into the mammary fat pad of Balb/c immunocompetent mice, and treatment was initiated when tumors reached 75 to 100 mm³. Treatment with anti-EMP2 mAbs significantly reduced primary tumor load compared with those treated with control mAbs (Fig. 6B; two-way ANOVA, \( P = 0.002 \)), and a reduction in tumor weight was also computed following treatment with the anti-EMP2 mAb compared with control untreated mice (\( P = 0.002 \)). To further interrogate these results, metastatic burden was assessed using the ALDEFLUOR assay on the primary tumor, and lung nodules were enumerated manually by counting 4 x fields from 5 tumors (Fig. 6C and D). Treatment with anti-EMP2 mAbs resulted in a marked reduction in lung metastasis, and representative images of lung metastasis posttreatment with either the Ctrl or anti-EMP2 mAbs are shown in Fig. 6C. Enumeration of nodules showed a greater than 2-fold reduction in tumor lesions in the lung (\( P = 0.004 \)) with an average of 25.8 ± 2.7 nodules counted following Ctrl mAb treatment compared with 11.5 ± 1.6 nodules following anti-EMP2 mAb treatment (Fig. 6D, top). We next correlated ALDH activity using the ALDEFLUOR assay with the number of lung nodules. Anti-EMP2 mAbs produced a significant reduction in ALDH activity, supporting previous studies that ALDH activity serves as a surrogate for metastatic potential (\( P = 0.003 \); Fig. 6D, bottom; refs. 8, 34). Collectively, our results identify EMP2 as an attractive therapeutic target and demonstrate that anti-EMP2 mAbs effectively inhibit the BCSC population reducing tumor initiation, growth, and metastasis across a spectrum of breast cancer models.

Discussion

Over the past decade, significant developments have furthered the understanding of intra- and interlesional tumor cell heterogeneity. Contributing to this heterogeneity are cellular hierarchies, which resemble those established during normal development and organogenesis. At the apex of these hierarchies are “stem like” cells that may drive tumor initiation and metastasis. These cells may also contribute to the resistance of tumors to a variety of cancer therapeutic agents (4, 6, 33), highlighting the need to identify novel therapeutic approaches capable of targeting this cell population. In these studies, we identify EMP2 as an important regulator of BCSCs and demonstrate that EMP2 genetic knockdown or inhibition with a mAb reduces the proportion of CSC in vitro and in vivo using mouse xenograft models. Although the mechanism of EMP2 regulation is not known, this study supports and extends the observation that EMP2 may help regulate HIF-1α expression (16), a transcription factor critical for the cellular response to hypoxia (21). We have recently demonstrated that HIF-1 regulates ALDH1+ BCSCs through an AMPK-dependent pathway (35). Although our data demonstrate that EMP2 is upstream of HIF-1α and helps stabilize and/or promote its expression, it does not establish a clear causal relationship between the two.

Within breast cancer, EMP2 was predominantly expressed on the membrane and cytoplasm of the tumor parenchyma with low levels of protein observed in histologically “normal” adjacent mammmary tissue. In disease progression, no differences in EMP2 expression were observed in patients with IDC who also had foci diagnosed as DCIS. This is not completely unexpected as several studies have shown that the regions up to a 1 cm from the margin of the tumor display abnormalities relative to normal, tumor-bearing tissue (31). These results suggest that overexpression of EMP2 may be an early event in carcinogenesis, or it may simply reflect the similarities of high-grade DCIS with IDC.

ALDH1 expression, in contrast, displayed a heterogeneous staining pattern within the tumor parenchyma, with tumor nests visualized in some patients. Although all ALDH1-positive tumor cells were EMP2 positive, no clear correlation between EMP2 and ALDH1 expression intensity occurred within patients’ primary tumors. In contrast to this, EMP2 and ALDH1 expression correlated within metastatic lymph nodes of patients. Several possibilities may account for this apparent discrepancy. First, we have reported that although ALDH1 is a marker of epithelial proliferative BCSCs, more mesenchymal BCSCs are characterized by CD44+/CD24− expression (10). BCSCs maintain the plasticity to transition between these states in a process regulated by the tumor microenvironment (25). Thus, it may simply be that the tumors analyzed were captured in a mesenchymal state. A second possibility is that ALDH1 expression is insufficient to independently characterize CSCs. Historically, ALDH1A1 and ALDH1A3 have both been associated in BCSC identification (36), although in recent years, many other family members such as ALDH2, ALDH3A1, ALDH5A1, and ALDH7A1 have been implicated as well (37). Consistent with this possibility, in SUM149, differential mRNA expression was observed in 10 different ALDH isoforms. A final possibility may involve temporal regulation for ALDH1 in disease progression, with its induction occurring after the upregulation of EMP2. This possibility would be consistent with reports that ALDH1 expression correlates with larger tumor size (38).

One defining feature of BCSCs is therapy resistance, and in this regard, data presented here and in the literature suggest that EMP2 may play a significant role in this process. Several studies have documented EMP2 expression in endocrine-resistant (39), Adriamycin-resistant (40), as well as dasatinib-resistant tumors (41). Moreover,
Figure 6.

Anti-EMP2 therapy reduces tumor growth and metastasis. A, To create a model of metastasis, MDA-MB-231/Luc- cells were injected into the left ventricle of Balb/c nude mice, and tumor load was determined using bioluminescence. Mice were treated systemically, beginning at day 5, with 10 mg/kg anti-EMP2 IgG1 or control human IgG. Data were analyzed using the maximum photon flux emission (photons/second) throughout the whole animal. n = 4, *P < 0.05 as determined by Student t test. B, A total of 5 × 10^4 4T1-FLUC cells were implanted into the mammary fat pad of Balb/c immunocompetent mice and treated twice a week with 10 mg/kg control human mAbs or anti-EMP2 mAb starting on day 7 when tumors approached 75 to 100 mm^3. The arrow demarks treatment initiation, with grouped and individual data shown. n = 8. Two-way ANOVA, P = 0.002. **Significant by Bonferroni posttest. Right, animals were sacrificed at day 24 and tumors weighed. Mean tumor weight for the control mAb treated group was 1.5 ± 0.3 while the mean tumor weight for the anti-EMP2 mAb was 0.95 ± 0.1 (P = 0.04, Mann–Whitney test). n = 8. C, To assess pulmonary metastases, lungs were removed and stained with hematoxylin and eosin. Representative images are shown. Magnification, × 400. D, Top, enumeration of tumor nodules per 4 × field, P = 0.004, Student t test. Below, for half of the animals, ALDH activity was measured using the ALDEFLUOR assay in the remaining primary tumor. Average results are shown from 4 animals ± SEM, with significance determined using Student t test.
this study furthers these associations as GSEA suggests an association between clinical upregulation of EMP2 and invasion, stemness, and metastasis. Critically, anti-EMP2 mAbs inhibited many of the defining features associated with these cells. Anti-EMP2 therapy significantly reduced a number of in vitro markers associated with BCSCs, including tumorsphere formation and ALDH activity. Moreover, anti-EMP2 mAbs significantly reduced primary tumor formation in both primary tumorsgrafts and cultured cell lines. As these mAbs cross react with murine EMP2 (13), syngeneic systems were also created to determine whether the immune system can enhance or inhibit these effects. Similar to the results observed in immunocompromised models, anti-EMP2 mAbs inhibited tumor growth and specifically suppressed markers associated with stemness including ALDH1. Finally, anti-EMP2 mAbs inhibited tumor metastasis as a significant reduction in pulmonary metastasis were observed. Collectively, given the lack of toxicity observed to date with anti-EMP2 mAbs in animal models, these results support the continued development of EMP2 inhibitors in breast cancer.

In conclusion, our data support the role of EMP2 in BCSC formation. Additional experiments will be required to elucidate the mechanisms that regulate EMP2 expression as well as how it regulates BCSCs. Nevertheless, our results suggest that EMP2 may serve as a novel therapeutic target and that anti-EMP2 therapies may provide a novel means for BCSC elimination.

Disclosure of Potential Conflicts of Interest

M. Wadehra, J. Braun, and L.K. Gordon have ownership interests (including being inventors on the UCLA patents) in the anti-EMP2 antibodies described. M.W. was a scientific advisor to Onco_response. MSW has financial holdings and is a scientific advisor for OncoMed Pharmaceuticals, Verastem, and MedImmune and receives research support from Dompe Pharmaceuticals and MedImmune. No potential conflicts of interest were disclosed by the other authors.

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Writing, review, and/or revision of the manuscript: C. Dillard, M. Kiyohara, Y. Elshimali, P. Dhawan, L.K. Gordon, J. Braun, G. Lazar, M.S. Wicha, M. Wadehra
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Kiyohara, A.M. Chan, Y. Elshimali, Y. Wu, J.V. Vadgama, M. Wadehra
Study supervision: M. Wadehra

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EMP2 Is a Novel Regulator of Stemness in Breast Cancer Cells

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