Title: Hormonal regulation of Semaphorin 7a in ER+ breast cancer drives therapeutic resistance

Authors: Lyndsey S Crump[1,2], Garhett Wyatt[3], Jennifer K Richer[4], Weston W Porter[3], and Traci R Lyons[1,2,5]

Affiliations: [1]Division of Medical Oncology, Department of Medicine, School of Medicine, University of Colorado Anschutz Medical Campus, Aurora, CO, USA; [2]Young Women's Breast Cancer Translational Program, University of Colorado Anschutz Medical Campus, Aurora, CO 80045, USA. 3Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, TX, USA; [4]Department of Pathology, School of Medicine, University of Colorado Anschutz Medical Campus, Aurora, CO, USA; [5]University of Colorado Cancer Center, Aurora, CO, 80045, USA

Running Title: Semaphorin 7a in ER+ breast cancer

Keywords: breast cancer, semaphorin 7a, endocrine therapy, resistance

Additional Information:

Financial Support: This work was supported by Colorado Clinical and Translational Sciences Institute Grant TL1 TR001081 (to L.S.C.), National Institutes of Health Grants F31 CA236140 (to L.S.C.), NIH/NCI R01 CA211696-01A1 (to T.R.L.), NIH/NICHD R01-HD083952-01 (to W.W.P), NIH/NCI R01 CA187733-01 (to J.K.R), NIH/NCI R21-CA197896 (to W.W.P), DOD BCRP W81XWH-16-1-0422 (to J.K.R), ACS RSG-16-171-010CSM (to T.R.L.), and Department of Medicine Outstanding Early Career Scholars Award (to T.R.L.).

Corresponding author: Traci R Lyons, University of Colorado Anschutz Medical Campus, Department of Medicine/Medical Oncology, MS8117, RC1S-8106, 12801 East 17th Ave, Aurora, CO 80045, traci.lyons@cuanschutz.edu

Disclosure Summary: The authors have nothing to disclose.

Word count: 6,067

Number of figures: 7

Number of tables: 0

ABSTRACT

Approximately 70% of all breast cancers are estrogen receptor positive (ER+BC) and endocrine therapy has improved survival for ER+BC patients. Yet, up to half of these tumors recur within 20 years. Recurrent ER+BCs develop resistance to endocrine therapy; thus, novel targets are needed to treat recurrent ER+BC. We found that semaphorin 7A (SEMA7A) confers significantly decreased patient survival rates in ER+BC. We show that *SEMA7A* is hormonally regulated in ER+BC, but its expression does not uniformly decrease with anti-estrogen treatments. Additionally, overexpression of SEMA7A in ER+ cell lines drives increased *in vitro* growth in the presence of estrogen-deprivation, tamoxifen, and fulvestrant. In *in vivo* studies, we found that SEMA7A confers primary tumor resistance to fulvestrant and, importantly, induced lung metastases. Finally, we identify pro-survival signaling as a therapeutic vulnerability of ER+SEMA7A+ tumors and propose that targeting with inhibitors of survival signaling such as PI3K inhibitors and venetoclax may have efficacy for treating SEMA7A+ tumors.

SIGNIFICANCE

Identifying patients who will experience disease relapse and developing therapeutic strategies to treat recurrent, metastatic ER+ breast cancer is critical. We report that SEMA7A predicts for, and likely contributes to, poor response to standard-of-care therapies and suggest that patients with SEMA7A+ER+ tumors may benefit from alternative therapeutic strategies.

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer-associated death in women in the United States. Estrogen receptor positive (ER+) breast cancers comprise approximately 70% of all breast cancer cases. The development of effective anti-estrogen-based endocrine therapies, including aromatase inhibitors and selective estrogen receptor modulators (e.g., tamoxifen) or degraders (e.g., fulvestrant) has facilitated the successful treatment of ER+ primary tumors. However, depending on nodal involvement, 22-52% of women with ER+ breast cancer will experience disease recurrence within 20 years (1), frequently in a distant metastatic site, such as lung, liver, bone, or brain. These recurrent ER+ breast tumors uniformly develop resistance to endocrine therapies, rendering most therapeutic interventions in the clinical setting ineffective at preventing metastatic outgrowth. Therefore, novel molecular targets are needed to predict and treat recurrent and/or metastatic ER+ breast cancers.

Semaphorin 7a (SEMA7A) is a glycosylphosphatidylinositol-anchored member of the semaphorin family of signaling proteins. SEMA7A has well-described roles in the promotion of axon guidance, immune modulation, and cellular migration (2-6). In adults, SEMA7A is expressed in the brain, bone marrow, lung, and skin, but is minimally expressed in many adult tissues, including the mammary gland (information via https://www.proteinatlas.org). Interestingly, in neuronal cells of the hypothalamus, SEMA7A expression is regulated by the steroid hormone progesterone via the progesterone receptor (PR) (7), while in pulmonary fibrosis models, SEMA7A is regulated by TGF-β (8). In its normal physiological role, SEMA7A binds β1-integrin to activate downstream signaling cascades, including pro-invasive MAPK/ERK and pro-survival PI3K/AKT pathways (2, 5, 8). Our group and others have described tumor-promotional roles for SEMA7A in mammary cancers, including tumor and host derived mechanisms of increased tumor growth, cellular migration, epithelial to mesenchymal transition, immune cell infiltration, remodeling of the blood and lymphatic vasculature, and metastatic spread (9-13). More recently, it has been reported that SEMA7A expression in lung cancer confers resistance to EGFR tyrosine kinase inhibition (14).

Studies to date have investigated the role of SEMA7A in breast cancer using pre-clinical models of triple negative breast cancer (TNBC), which lack expression of ER (ER-), PR (PR-), and amplification of HER2 (HER2-). Specifically, our studies in TNBC reveal that SEMA7A promotes TNBC cell growth, invasion, lymphangiogenesis, lymph vessel invasion, and metastasis (10, 15). However, our previous genomics analysis in patient datasets demonstrated that *SEMA7A* expression correlates with poor prognosis in both ER- and ER+ patients and that *SEMA7A* expression levels were highest in luminal A tumors, which are generally ER+ and/or PR+ and HER2-) (9). Thus, understanding the role of SEMA7A in ER+ and/or PR+ breast cancers is critical for a complete understanding of its role and impact on breast cancer progression. Our results presented herein show that *SEMA7A* mRNA expression is highest in patients with ER+ breast cancer and that SEMA7A induces pleiotropic tumor-promotional effects in ER+ breast cancer cells. We also describe a novel relationship between hormone signaling and expression of SEMA7A in breast cancer. Additionally, we report for the first time that expression of SEMA7A confers resistance to standard-of-care endocrine therapy and to CDK 4/6 inhibition in pre-clinical ER+ models. Finally, using pre-clinical models, we identify a therapeutic vulnerability of SEMA7A-expressing tumors with the BCL2 inhibitor venetoclax (ABT-199/GDC-0199). These data provide promising evidence that SEMA7A+ER+ breast cancers that do not respond to endocrine therapies may selectively respond to novel treatment regimens in the clinic.

MATERIALS AND METHODS

Cell culture

MCF7 cells were obtained from K. Horwitz (University of Colorado Anschutz Medical Campus.) T47D and BT474 cells were obtained from V. Borges and M. Borakove (University of Colorado Anschutz Medical Campus). E0771 cells were purchased from CH3 Biosystems (Amherst, NY). MCF10DCIS cells were obtained from K. Polyak and A. Marusyk (Harvard University, Cambridge, MA). MDA-MB-231 cells were obtained from P. Schedin (Oregon Heath and Sciences University, Portland OR). Cell lines were validated by fingerprinting analysis at the University of Colorado Anschutz Medical Campus Molecular Biology Core. Cells were routinely

tested for mycoplasma contamination throughout the studies described herein with either Lonza MycoAlert PLUS (Walkersville, MD) or Mycoflour Mycoplasma Detection Kit (Molecular Probe, Eugene OR). Cells were cultured at 37 °C and 5% CO2. MCF7 and BT474 cells were grown in MEM with 5% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin/streptomycin (P/S; ThermoFisher Scientific). T47D and E0771 cells were grown in RPMI 1640, with 10% heat-inactivated FBS and 1% P/S. Experiments were performed with cells under passage 30. Forced suspension cultures were performed by coating plates with 12 mg/ml poly-2-hydroxyethyl methacrylate (Sigma, St. Louis, MO). For exogenous SEMA7A treatments, SEMA7A was purified from SEMA7A-Fc expressing MDA-MB-231 cells in collaboration with the CU Anschutz Medical Campus Protein Purification/MoAB/Tissue Culture Core.

Transfections

For knockdown studies, SEMA7A-targeting shRNA plasmids (shSEMA7A KD1 and KD2) or a scrambled control (Scr; SA Biosciences, Frederik, MD, and the Functional Genomics Facility at the CU Anschutz Medical Campus) were transfected using X-tremeGENE transfection reagent (Millipore Sigma, St. Louis, MO) as described by the manufacturer. SEMA7A overexpression in MCF7 cells was achieved using a SEMA7A-Fc plasmid (generous gift from R. Medzhitov, Yale University, New Haven, CT). A pcDNA3.1 empty vector control plasmid was obtained from H. Ford (CU Anschutz Medical Campus). All other plasmids were obtained from the Functional Genomics Core at the University of Colorado Anschutz Medical Campus. E0771 cells were transfected with either DDK-tagged Sema7a or DDK empty vector control plasmids. Knockdown and overexpression were confirmed via qPCR and Western blot analysis.

Luciferase Reporter Assay

The reporter construct was generated by cloning a -820 to +182 base pair fragment of the *SEMA7A* promoter into the pGL3-Basic Luciferase reporter (Invitrogen). 50,000 cells were seeded in a 12-well plate and incubated for 5 minutes with 50µL Opti-MEM (Invitrogen, Carlsbad, CA) and 3µL GeneJuice (Millipore Sigma, Burlington, MA) per well. 0.5-1µg of DNA was added to GeneJuice Transfection Reagent/serum-free medium mixture. Cells were harvested 48 hours after transfection using Reporter Lysis Buffer (Promega, Madison, WI), unless treated with 10nM E2 and/or 100nM P4. If treated with E2 and/or P4, cells were serum

starved for 48 hours in serum-free medium. Cells were then treated with 10nM E2 and/or 100nM P4 for 48 hours and harvested as stated above. Luciferase activity was normalized to total protein.

Drug and Hormone Treatments

Fulvestrant, 4-hydroxytamoxifen, mifepristone, progesterone (P4), and 17-β estradiol (E2) were purchased from Millipore Sigma (St. Louis, MO). For *in vitro* hormone treatments, cells were cultured in charcoal-stripped serum (Thermo Scientific, Waltham, MA) containing phenol red-free media for 3-5 days, then treated with 10nM E2 and/or 100nM P4. Fulvestrant, tamoxifen, and mifepristone treatments were at a final concentration of 7.5nM, 5μM, and 40nM, respectively. Palbociclib and SU6656 were acquired from MedChemExpress (Monmouth Junction, NJ) and treatments were at a final concentration of 2.5μM and 5μM, respectively, in DMSO. LY294002 was purchased from Cell Signaling Technologies (Danvers, MA) and *in vitro* treatments were at a final concentration of 2.5μM LY294002 in DMSO. *In vitro* venetoclax (Abbvie, Chicago, IL) was at a final concentration of 300nM in DMSO.

Cell Growth, Invasion, and Death Assays

100,000 MCF7 cells were seeded per well of a 6-well dish to assess growth *in vitro*. Cells were fixed with 10% NBF and stained with crystal violet at the indicated time points. 1,000 MCF7 cells or 5,000 T47D cells were seeded per well of a 6-well dish and assessed 7-14 days later for clonogenic assays. 3D matrigel and collagen cultures were performed as previously described (9, 16). Cleaved caspase 7 was measured using a Caspase Glo assay (Promega, Madison, WI) as described by the manufacturer. Exogenous SEMA7A treatments were at a final concentration of 5μg/mL.

RNA isolation

RNA was isolated using the commercially available Quick-RNA kit (Zymo Research, Irvine, CA). RNA concentration and purity were assessed using a Nanodrop machine via absorbance at 280 nm, 260 nm, and 230 nm. 1μg of RNA was used to synthesize cDNA with the Bio-Rad (Hercules, CA) iScript cDNA Synthesis kit with the following conditions: 25ºC for 5 min., 42ºC for 30 min., and 85ºC for 5 min.

mRNA and protein expression analysis

cDNA was quantified via qPCR using Bio-Rad iTaq Universal SYBR Green Supermix and primers for *SEMA7A* (Bio-Rad PrimePCR, Hercules, CA) using the following conditions: 95ºC for 3 min, then 40 cycles of 95ºC for 15 sec and 60ºC for 1 min, 95ºC for 30 sec, and 55ºC for 1 min. Primer fidelity was assessed for each qPCR experiment using a 95ºC melt curve. *SEMA7A* mRNA was detected with Bio-Rad primers (forward: CTCAGCCGTCTGTGTGTATT, reverse: CTCAGGTAGTAGCGAGAGTTTG). Primers for *ESR1 (forward: 5-GAACCGAGATGATGTAGCCA, reverse: GTTTGCTCCTAACTTGCTCTTG)* and *PGR (forward: ATTTCATCCACGTGCCTATCC, reverse: CCTTCCTCCTCCTCCTTTATCT)* were purchased from IDP. mRNA quantification was normalized using the geometric average of two reference genes: *GAPDH* (forward: CAAGAGCACAAGAGGAAGAGAG, reverse: CTACATGGCAACTGTGAGGAG) and *RPS18* (forward: GCGAGTACTCAACACCAACA, reverse: GCTAGGACCTGGCTGTATTT). Data were analyzed using the relative standard curve method.

Western blots were performed by loading equal amounts of protein lysates as determined by Bradford assay. Samples were separated on a 10% tris-glycine SDS polyacrylamide gel and electroblotted onto a polyvinylidene fluoride membrane. SEMA7A blots were probed with anti-SEMA7A (clone C6, Santa Cruz Biotechnologies, Dallas, TX) and goat-anti-mouse secondary (Abcam, Cambridge, MA). Blots were then exposed and developed on film. Color images were converted to black and white for presentation, and, if applied, color corrections were applied uniformly to all lanes of the blot to ease viewing of bands. No nonlinear intensity or color adjustments to specific features were performed.

Animal models

2,500,000 SEMA7A overexpressing or empty vector control MCF7 cells were injected into the #4 mammary fat pads of 6-7 week old female NSG mice (Jackson Laboratory, Bar Harbor, ME) or NCG mice (Charles River, Wilmington, MA). Tumors were measured using digital calipers every 3-4 days, and tumor volume was calculated as (L x W x W x 0.5). Fulvestrant treatments began when average tumor volume reached ~200mm^2 with the following dosing regimen: 2.5mg fulvestrant in 10% ethanol and 90% sesame oil, injected subcutaneously every 5 days. This dose is equivalent to approximately 83mg/kg per mouse and, using the FDA

Dosing Guidance for Industry: Conversion of Animal Doses to Human Equivalent Doses, equivalent to 6.7mg/kg in humans. For venetoclax studies, venetoclax was dissolved in phosal-50G:polyethylene glycol-400:ethanol at 6:3:1 to a final concentration of 12mg/mL and administered via oral gavage. Tumors were harvested at designated study endpoints. Mammary glands with intact tumor and lungs were harvested, formalin fixed, and paraffin embedded for immunohistochemical analysis.

For experimental metastasis studies, 500,000 E0771 DDK control or DDK-Sema7a overexpression cells were suspended in 50uL PBS and injected into the tail veins of C57Bl/6 mice. Animals were euthanized 10 days post-injection. Lungs were harvested, and the number of metastatic tumor lesions were quantified per lung lobe per animal. All animal studies were performed under the approval of the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee.

Immunohistochemistry

Tissues were formalin-fixed and paraffin-embedded as previously described (17). Antibody information is provided in STable 1. For staining quantification, slides were scanned into the Aperio Image Analysis software (Leica, Buffalo Grove, IL). The total tumor area was quantified by removing necrotic and stromal areas. Custom algorithms for specific stains were used to quantify stain intensity or percent positive stain as assessed by percent weak, medium, and strong determined using the color-deconvolution tool.

Data Mining Analyses

Kaplan Meir Plotter (18) was used to assess relapse-free survival (RFS) data for 5,143 breast cancer patients with mean follow-up of 69 months. SEMA7A was queried and stratified into high and low expression groups using the auto select best cutoff level function. The generated data were exported, graphed, and analyzed in Graphpad Prism to calculate hazard ratio (HR) with 95% confidence intervals and log rank P values. The Gene Expression Based Outcome for Breast Cancer Online platform (http://co.bmc.lu.se/gobo/) was used to query *SEMA7A* expression in 1,881 available sample tumors and a panel of 51 breast cancer cell lines. Data were stratified and reported by molecular subtype. Outcomes data were reported in ER+ breast cancer patients only. Gene expression data for *SEMA7A* and clinical response to anti-

endocrine therapy was extracted from publically available datasets GSE59515 and GSE20181 provided by Turnbull et al. (19). Data were stratified by clinical response (responders vs non-responders) and graphed by timepoint. Linear regression was performed for each group using Graphpad Prism. The Oncomine Platform (https://www.oncomine.org) was used to query *SEMA7A* in available breast cancer datasets. The p-value threshold was set to 0.5. All fold-changes and gene ranks were accepted. Data are reported from the molecular subtype analysis, correlation with stage and grade, and drug sensitivity screens.

IncuCyte Assay

5,000 SEMA7A overexpressing or empty vector control MCF7 cells were seeded per well in a 96 well plate and allowed to adhere overnight. Media was replaced with fresh drug or vehicle treated media the next morning. Drug treatments included 7.5nM fulvestrant, 5μM 4-hydroxytamoxifen, 2.5μM palbociclib, 10μM LY294002, 5μM Su6656, and growth was assessed in an IncuCyte live cell analysis system at the University of Colorado Cancer Center Tissue Culture Core. Confluence was reported every 4 hours and representative videos were chosen.

Statistical analysis

All cell line and xenograft experiments were performed in biological duplicate or triplicate. Data are expressed as means ± SEM for animal studies and ± SD for *in vitro* studies. All statistical analyses were performed on raw data. An unpaired two-tailed student's t test was performed for analyses comparing two groups, while a one-way ANOVA followed by a Tukey–Kramer test was used for multiple comparisons of groups of 3 or more. For tumor volume data and incucyte analyses, a repeated measures one-way ANOVA was used. For Kaplan–Meier analyses, the p-value was determined using a log-rank test. All p-values less than or equal to 0.05 were considered statistically significant, and all statistical analyses were performed in GraphPad Prism 7.01 (San Diego, CA).

RESULTS

SEMA7A is expressed in ER+ breast cancers and confers poor prognosis

To determine if SEMA7A is expressed in ER+ breast cancer, we assessed *SEMA7A* mRNA expression using the Gene Expression-based Outcome for Breast Cancer Online (GOBO) tool. We observed that 67% of luminal subtype cell lines, which are typically ER+, exhibit increased *SEMA7A* expression versus 36-50% of the basal subtypes (which are typically ER-; Figure 1A). Average expression was higher when ER+ cell lines were compared to TNBC cell lines and when luminal cell lines were compared to basal subtype (Figure 1B). *SEMA7A* mRNA expression is also increased in ER+ patient tumors (Figure 1C), and we confirmed that *SEMA7A* was overexpressed and/or had a higher copy number rank in ER+ or PR+ disease in 7 additional datasets using Oncomine (STable 2). Additionally, *SEMA7A* was highest in stage III and IV, grade 2 and 3, and lymph node positive (LN+) disease in multiple datasets (STable 3). We next examined a role for *SEMA7A* status in predicting ER+ patient outcomes and observed that patients with high *SEMA7A* expression had decreased overall and distant metastasis free survival (Figure 1D-E and SFigure 1A-B). Additionally, using KM Plotter we further stratified relapse-free survival by LN positivity in the ER+PR+(HER2+/-) subset and observed that high SEMA7A is associated with decreased relapse free survival in both LN+ and LN- patients (Figure1F). In fact, the hazard ratio for ER+PR+(HER2+/-) SEMA7A+LN- patients was the highest. Additionally, in a small protein expression dataset, we observed a trend toward decreased overall survival with high SEMA7A expression (SFigure 1C). We then confirmed SEMA7A protein expression in ER+ lines (MCF7, T47D, BT474), ER- lines (DCIS, MDA-MB-231, SUM159), and immortalized normal mammary epithelial cells (MCF10A) via immunoblot analysis. SEMA7A expression was observed in two of the three ER+ cell lines and one of the three ER- when compared to the MCF10A cells (SFigure 1D).

***SEMA7A* is regulated by hormone signaling in breast cancer cells and correlates with relapse in patients with ER+ breast cancer**

To determine whether *SEMA7A* expression is promoted by ER and/or PR in breast cancer cells, we performed an *in silico* analysis of the *SEMA7A* promoter using the ConTra (conserved transcription factor binding site) web server (20). We identified two highly conserved estrogen response element (ERE) half sites and one progesterone response element half site (Figure 2A). We then utilized a *SEMA7A* luciferase reporter construct to measure promoter activity with E2 treatment and observed increased luminescence with 10nM

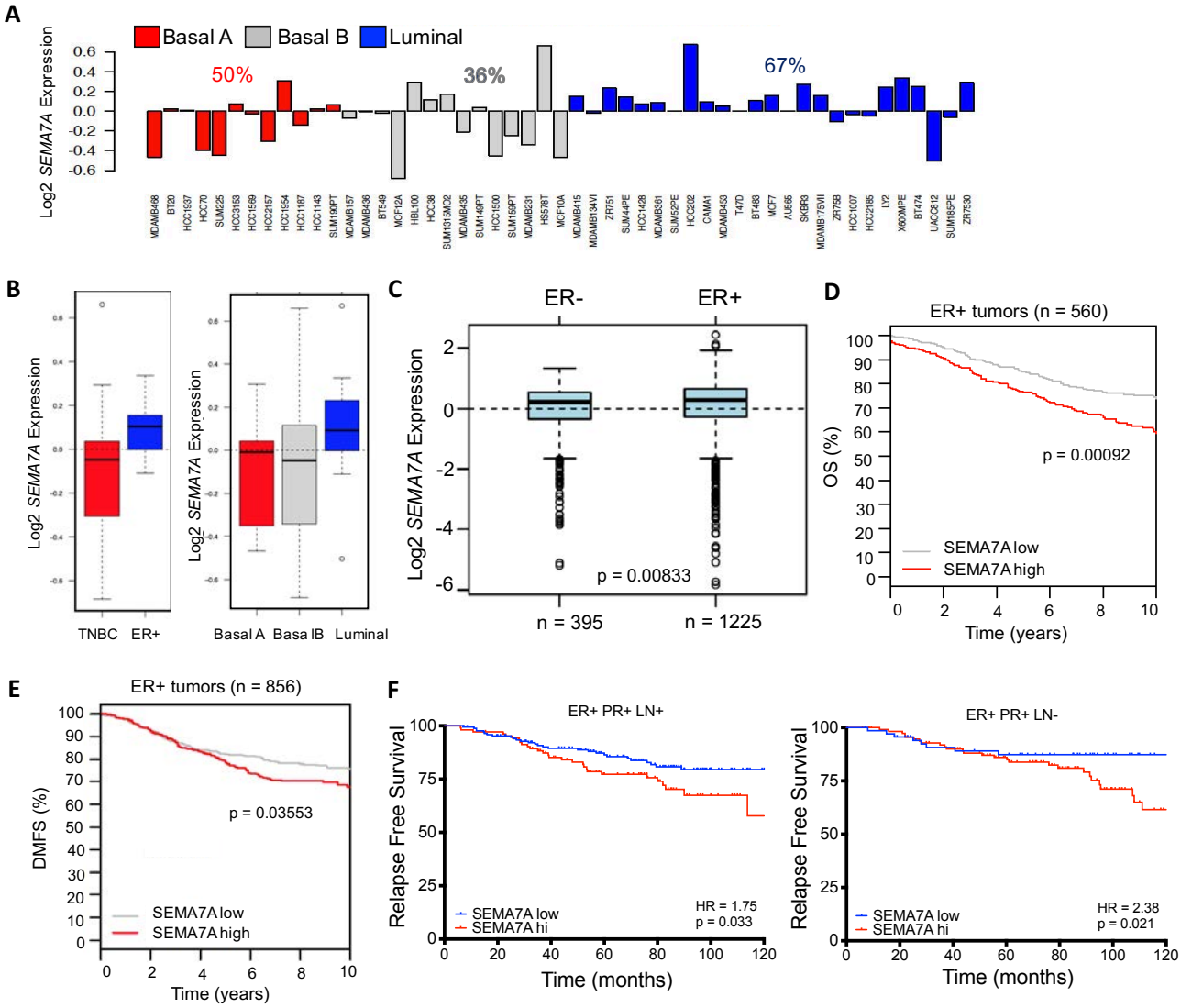

Figure 1: *SEMA7A* is expressed in ER+ breast cancer and associated with poor prognosis. (A-B) The Gene Expression-Based Outcome dataset was assessed for *SEMA7A* mRNA expression levels in basal/triple negative breast cancer (TNBC) and luminal/ER+ breast cancer cell lines (n = 51) and **(C)** in ER- or ER+ patient tumors (n = 1620). In the ER+ subset, **(D)** overall survival (OS) and **(E)** distant metastasis free survival (DMFS) curves are stratified by *SEMA7A* high (red) and low (grey) expression. **(F)** Relapse free survival curves for patients with ER+ and PR+ disease and either lymph node positivity (LN+; n = 277) or negativity (LN-; n = 185) are stratified by high *SEMA7A* (red) and low *SEMA7A* (blue) using KMplotter mRNA gene chip data. Hazard ratios (HR) are included.

E2 (Figure 2B). Next, we treated MCF7 cells with estradiol (E2) and/or progesterone (P4) after being grown in hormone depleted conditions, and we observed upregulation of SEMA7A protein and mRNA (Figure 2C and SFigure 2A). Interestingly, SEMA7A levels were highest in our dual treated (E2+P4) cells, and fulvestrant, a selective ER degrader (SERD), was not sufficient to completely block expression of SEMA7A under normal growth conditions (Figure 2D). Since MCF7 cells are ER+PR- cells that can be induced to express PR protein with estrogen treatment, we sought to determine whether protein synthesis is required in MCF7 cells for ER-mediated expression of SEMA7A. We treated MCF7 cells with E2 and P4 in the presence or absence of cycloheximide (CHX), a protein synthesis inhibitor, to show that CHX treatment blocked E2/P4 mediated SEMA7A expression (Figure 2E). To test if the addition of P4 to E2 treatment would also increase *SEMA7A* promoter activity, we utilized our luciferase reporter construct and observed increased luminescence with dual treatment of E2 and P4 (Figure 2F). Next, we treated T47D cells, which are ER+PR+, with P4 alone and with an inhibitor of PR, mifepristone, to show that SEMA7A expression can also be induced by P4 alone and partially blocked by inhibition of PR (Figure 2G). Further, we observed that only dual ER and PR inhibition was sufficient to completely block SEMA7A expression when E2+P4 are present (Figure 2H). Collectively, these results suggest that both ER and PR can regulate expression of *SEMA7A*.

To determine whether *SEMA7A* gene expression in breast cancers correlates with expression of the genes encoding for ER or PR, *ESR1* and *PGR*, we examined the METABRIC dataset, which contains gene expression data from over 2,000 patients. We found that *SEMA7A* did not significantly correlate with *ESR* expression ($p = 0.6043$), but did significantly correlate with *PGR* expression ($p = 0.0253$; SFigure 2B). Additionally, to determine whether *SEMA7A* alters relapse free survival rates differentially based on ER or PR status, we stratified our Kaplan Meir analysis of relapse free survival into patients with ER+PR+, ER+PR- or ER-PR+ tumors; interestingly, the effect of *SEMA7A* expression on promotion of relapse is mostly negated when the PR+ patients are removed, and is highest in ER-PR+ breast cancer with a hazard ration of 5.35, although this did not reach statistical significance, likely due to the $n = 22$ patients (Figure 2I).

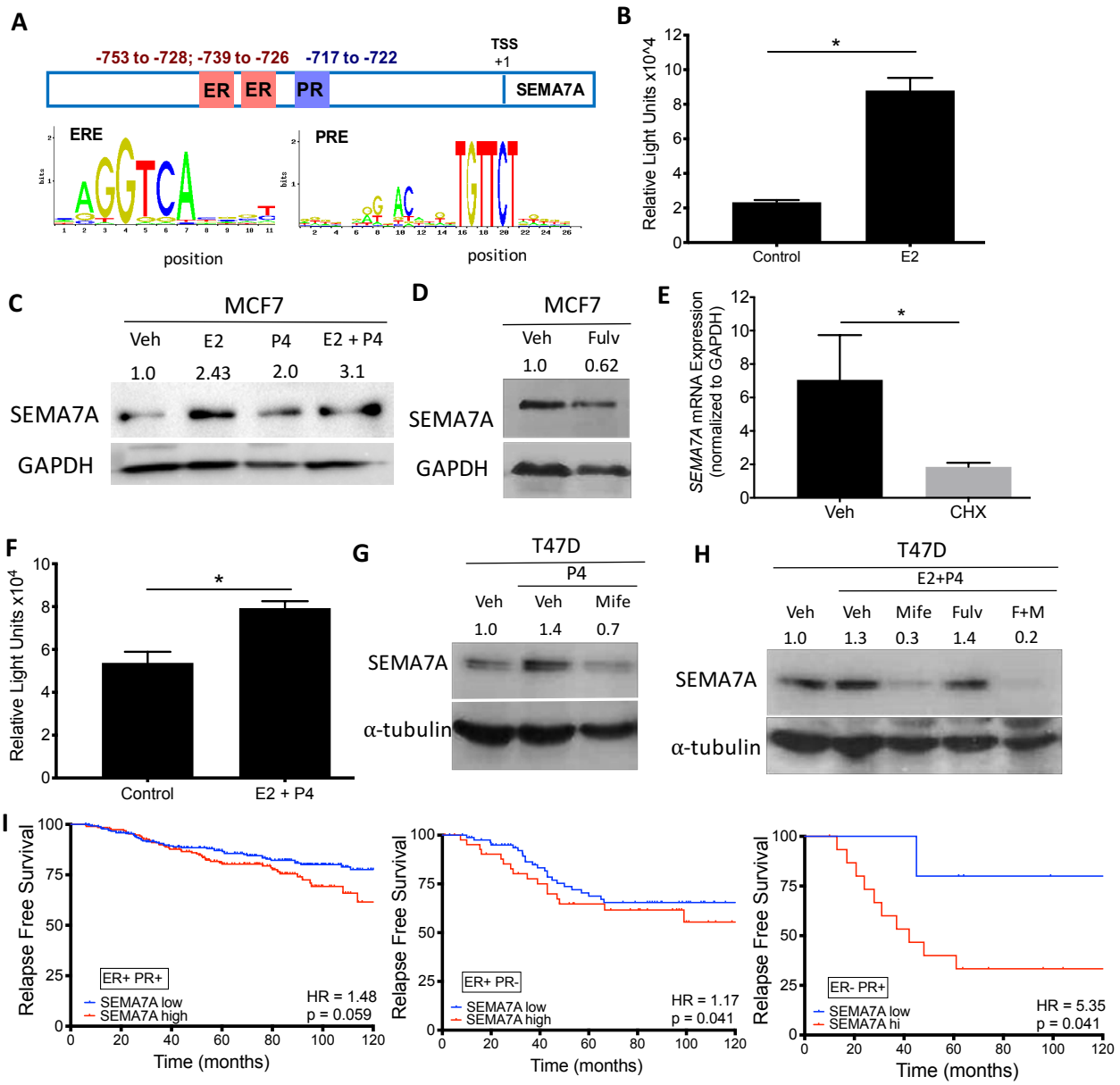

Figure 2: Expression of *SEMA7A* is hormonally regulated. (A) A ConTra analysis revealed multiple conserved ER and PR response elements (ERE and PRE) in the *SEMA7A* promoter. **(B)** Luciferase activity in MCF7 cells transfected with a luciferase reporter containing the *SEMA7A* promoter upstream of the luciferase gene, then treated with 10nM estrogen or vehicle control (n = 3). **(C)** MCF7 cells were charcoal-stripped for 72 hours, then treated with 10nM estrogen (E2) +/- 100nM progesterone (P4) or vehicle. Representative SEMA7A and GAPDH levels are shown via immunoblot. **(D)** MCF7 cells were treated with 7.5nM fulvestrant (Fulv) or vehicle. Representative SEMA7A and GAPDH levels are shown via immunoblot. **(E)** T47D cells were treated with cycloheximide (CHX) to inhibit protein synthesis, then *SEMA7A* was assessed by qPCR. Values are normalized to GAPDH and RPS18 (n = 3). **(F)** Luciferase activity in MCF7 cells transfected with a luciferase reporter containing the *SEMA7A* promoter upstream of the luciferase gene, then treated with 100nM E2 and 100nM P4 or vehicle control (n = 3) **(G)** T47D cells were treated with 40nM mifepristone (Mife) in the presence of P4 or **(H)** E2 and P4, with or without fulvestrant. **(I)** Relapse free survival curves for patients with ER+PR+ (left), ER+PR- (middle), or ER-PR+ (right) disease using KMplotter mRNA gene chip data. Hazard ratios (HR) are included. *p < 0.05, ***p < 0.005 by unpaired two-sided t-test (B, E, F)

SEMA7A induces growth and confers therapeutic resistance in ER+ breast cancer cells

To determine if SEMA7A promotes ER+ breast cancer progression via increased tumor growth, we generated two independent shRNA mediated knockdown (KD) variants of *SEMA7A* and confirmed decreased SEMA7A protein in MCF7 cells (Figure 3A). We observed that SEMA7A KD cells were less confluent over a 72 hour time course (Figure 3B). Similarly, SEMA7A KD cells exhibited decreased colony formation in a clonogenic assay (Figure 3C). We also show, via BrdU incorporation in a 3D organoid system (SFigure 2C), that SEMA7A KD organoids have significantly reduced proliferation (Figure 3D). Conversely, in cells overexpressing *SEMA7A,* where we confirmed 3.6-fold increase in SEMA7A protein (SEMA7A OE; Figure 3E), we observed increased cell growth in two-dimensional cultures (Figure 3F), clonogenic assays (Figure 3G), and 3D cultures (Figure 3H).

Given the relationship between SEMA7A, hormone receptors, and growth revealed by our studies, along with our data showing that patients with SEMA7A+ER+ breast cancers are more likely to relapse, we sought to determine if SEMA7A confers resistance to ER-targeting endocrine therapy. Kaplan Meir analysis of patients who received up to 5 years of treatment with tamoxifen revealed that high *SEMA7A* expression predicts for increased relapse (Figure 4A). Both tamoxifen and aromatase inhibitors, such as letrozole, are used to target ER+ tumors via competitive inhibition of ER or blockade of estrogen synthesis. We thus queried two additional independent analyses of *SEMA7A* gene expression data from patients treated with neoadjuvant letrozole (19), where sequencing data is available from a pre-treatment biopsy, at 2 weeks of treatment, and at 3 months of treatment. This revealed a trend towards increased *SEMA7A* expression (after 2 weeks and 3 months of treatment) in patients who did not respond to letrozole, whereas tumors that responded showed either a decrease or no change in *SEMA7A* levels (SFigure 3A-B). This suggests that uncoupling *SEMA7A* expression from estrogen regulation, such that it increases with the selective pressure of estrogen deprivation, facilitates resistance to letrozole.

To determine whether ER-targeting was ineffective for SEMA7A expressing cells, we cultured MCF7 SEMA7A OE cells in the presence of tamoxifen and fulvestrant and measured cell growth and survival compared to controls. We observed that SEMA7A OE cells grew

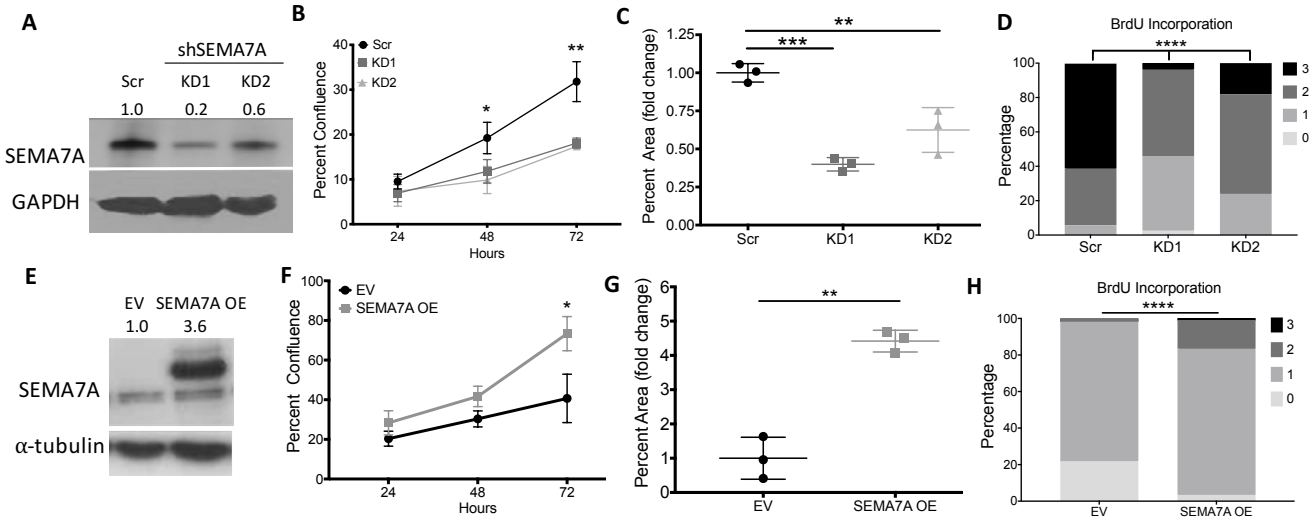

Figure 3: SEMA7A promotes growth of ER+ cells in 2D and 3D cultures. (A) Representative immunoblot for SEMA7A and GAPDH in MCF7 cell transfected with a with scrambled control (Scr) or shSEMA7A knockdown (KD1 and KD2) constructs. **(B)**. Confluence of Scr, KD1, or KD2 MCF7 cells at 24, 48, and 72 hours post seeding (n = 3). **(C)** Clonogenicity of Scr, KD1, or KD2 MCF7 cells. KD1 and KD2 values are normalized to Scr controls (n = 3). **(D)**. BrdU incorporation was assessed in Scr, KD1, or KD2 MCF7 cells embedded in Matrigel plus 20% collagen after 10 days in culture. Each organoid received a score 0-3 based on the percent positive of each organoid, where 0 = no BrdU+ cells, 1 = 1-49% BrdU+ cells, 2 = 50-99% BrdU+ cells, and 3 = 100% BrdU+ cells per organoid. **(E)**. Representative immunoblot for SEMA7A and GAPDH in MCF7 cell transfected with an empty vector (EV) control or SEMA7A overexpressing (SEMA7A OE) plasmid. **(F)** Confluence of EV or SEMA7A OE MCF7 cells at 24, 48, and 72 hours post seeding (n = 3). **(G)** Clonogenicity of EV or SEMA7A OE MCF7 cells. SEMA7A OE values are normalized to EV controls (n = 3). **(H)** BrdU incorporation was assessed in EV and SEMA7A OE MCF7 cells embedded in Matrigel plus 20% collagen after 5 days in culture. Scoring system as described for panel D. *$p < 0.05$, **$p<0.01$, ***$p < 0.005$, ****$p < 0.001$ by ANOVA with Tukey's multiple comparison test (B-D) or unpaired two-tailed t-test (F-H)

significantly better than EV controls with both drug treatments (Figure 4B-D; SFigure 3C-E). Of note, drug-treated SEMA7A OE cells grew as well as, or better than, vehicle-treated EV controls. Additionally, exogenous SEMA7A increased growth of both MCF7 and T47D cells, despite the presence of fulvestrant (Figure 4E; SFigure 3F). We also observed that MCF7 SEMA7A OE cells can grow in estrogen depleted conditions (SFigure 3G-H). To determine whether SEMA7A OE tumors would grow without estrogen supplementation *in vivo*, we orthotopically implanted SEMA7A OE cells and controls into mice that received estrogen supplementation or sham controls. Surprisingly, MCF7 SEMA7A OE tumors showed no initial growth benefit *in vivo* in animals with estrogen pellets. However, we did observe that SEMA7A OE cells form tumors *in vivo* in the absence of estrogen supplementation (Figure 4F). We next tested how SEMA7A OE tumors grow *in vivo* with endocrine therapy treatments. We allowed tumors to establish, then began administering fulvestrant when the tumors reached 200mm^3. Fulvestrant slowed the growth of control tumors, while the SEMA7A OE tumors continued to grow at the same rate (Figure 4G-H). At study completion, we verified SEMA7A overexpression by immunohistochemistry (IHC; SFigure 4A). Consistent with a resistant phenotype, our SEMA7A OE tumors exhibited increased proliferation as measured by Ki67 staining at study completion (Figure 4I; SFigure 4B) and were overall less necrotic (Figure 4J; SFigure 4C). Interestingly, ER+ tumor metastases frequently lose expression of ER under conditions of long-term estrogen deprivation (21), and our SEMA7A OE tumors exhibited decreased ER staining intensity by semi-quantitative IHC (Figure 4K; SFigure 4D), which could be a sign of ER independence and explain the lack of response to fulvestrant. Furthermore, *ESR1* and *PGR*—a known ER target gene—downregulation were confirmed in our MCF7 SEMA7A OE cells (Figure 4L). These results suggest that SEMA7A expression in ER+ breast cancer cells renders them less reliant on ER signaling for growth, which would also render them resistant to endocrine therapy and able to disseminate and seed metastases even in the presence of tamoxifen, aromatase inhibitors, or fulvestrant.

SEMA7A induces pro-metastatic phenotypes in ER+ breast cancer cells

Consistent with our previous results in ER- breast cancer cells (15), SEMA7A overexpression in ER+ MCF7 cells resulted in cells adapting a more mesenchymal morphology (SFigure 4E). To test whether SEMA7A also promotes invasion in ER+ cancer, we utilized the 3D model in

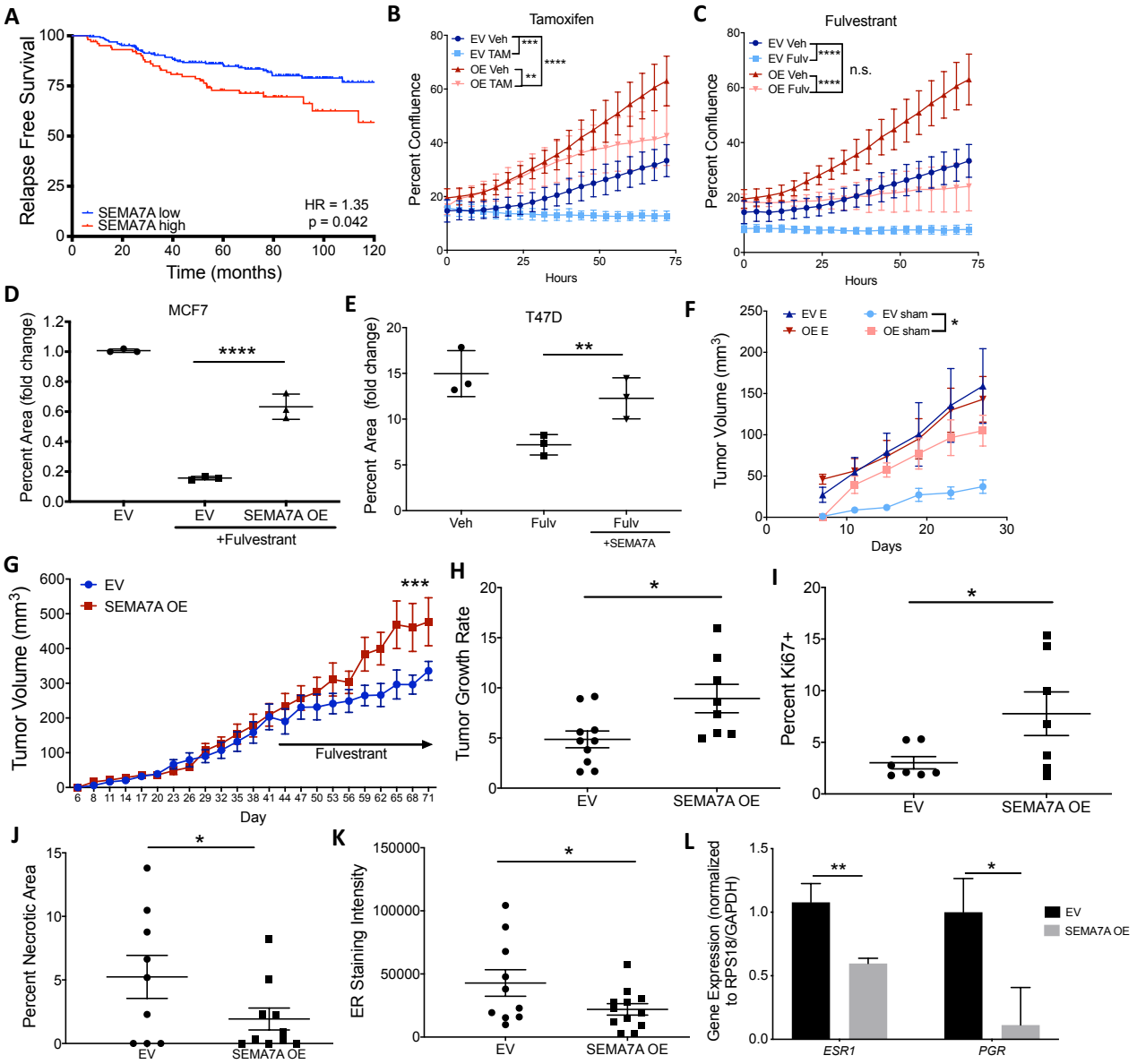

Figure 4: SEMA7A promotes endocrine resistance *in vitro* and *in vivo*. (A) Relapse free survival curves for patients with ER+ disease treated with tamoxifen are stratified by high *SEMA7A* (red) and low *SEMA7A* (blue) using KMplotter mRNA gene chip data. **(B)** Confluence of MCF7 EV or SEMA7A OE cells treated with 5μM 4-hydroxytamoxifen or **(C)** 7.5nM fulvestrant n = 3). **(D)** Clonogenic growth of EV or SEMA7A OE MCF7 cells treated with 7.5nM fulvestrant or vehicle. Fulvestrant-treated values are normalized to EV vehicle controls (n = 3). **(E)** Clonogenic growth of T47D cells treated with 7.5nM fulvestrant and 5μg/mL exogenous SEMA7A. Fulvestrant-treated values are normalized to vehicle controls (n = 3). **(F)** Tumor volumes for EV or SEMA7A OE MCF7 tumors in hosts with estrogen pellets (E) or sham controls (n = 6/group). **(G)** Tumor volumes for EV or SEMA7A OE MCF7 tumors in hosts treated with fulvestrant beginning on day 41 (n = 8-10/group). **(H)** Tumor growth rate (average change in tumor volume per day) of EV or SEMA7A OE MCF7 tumors on fulvestrant treatment. **(I)** Quantification of Ki67, **(J)** necrotic area, and **(K)** ER staining in EV or SEMA7A OE MCF7 tumors in hosts treated with fulvestrant. **(L)** qPCR for *ESR1* and *PGR* in EV or SEMA7A OE MCF7 cells (n = 3). *p < 0.05, **p<0.01, ***p < 0.005, ****p < 0.001 by unpaired t-test (D-E, H-L) or repeated measures one-way ANOVA with Tukey's multiple comparison test (B-C,F)

which we previously demonstrated that SEMA7A promotes invasion of ER- tumor cells (9, 16). Consistent with our hypothesis, SEMA7A OE cells exhibited evidence of invasion as early as 5 days post seeding, when most organoids in the control group remained in rounded, non-invasive spheroid structures (Figure 5A). Conversely, evidence of organoids with invasive protrusions appeared after 10 days in the control cells and were significantly less abundant in SEMA7A knockdowns (Figure 5B).

After local invasion and intravasation, metastatic tumor cells exit the primary site and survive in non-adherent conditions, which would induce death of normal epithelial cells via a process termed “anoikis” (22). Thus, we utilized forced suspension cultures to determine whether SEMA7A is upregulated in non-adherent conditions. We observed highest SEMA7A expression after 72 hours in suspension, suggesting that SEMA7A may be required for anoikis resistance (Figure 5C). To determine if SEMA7A expression promotes cell survival in forced suspension conditions, we measured cell death via cleaved caspase 7 to reveal decreased cell death in SEMA7A OE cells (Figure 5D). In contrast, SEMA7A KD cells showed a ~2-fold increase in cell death in both attached and forced suspension conditions (Figure 5E), suggesting that SEMA7A may mediate protection from cell death both at the primary site and in circulation during metastasis. Next, we analyzed lung tissues from our orthotopic xenografts (Figure 4G) to determine if human pan-cytokeratin+ tumor cells were more abundant in mice with SEMA7A OE tumors (Figure 5F). To analyze this mechanism using an additional experimental metastasis assay in immunocompetent mice, and to mimic downregulation of *ESR1* expression and gain of *SEMA7A* expression, we overexpressed *Sema7a* in the ER- murine mammary carcinoma cell line E0771 (10) and performed tail vein injections in estrogen supplemented mice. We observed that overexpression of murine *Sema7a* significantly increased metastatic burden in the lungs (Figure 5G). Collectively, these data suggest that Sema7a expression promotes cell survival in circulation, and potentially in the lung. This may be one mechanism by which SEMA7A promotes relapse in patients.

Therapeutic vulnerability of SEMA7A-expressing ER+ tumor cells

Based on our observation that SEMA7A drives metastasis of ER+ tumors and are resistant to endocrine therapy, we postulated that CDK 4/6 inhibitors, which have recently been approved and proven effective for patients with metastatic ER+ breast cancer, could inhibit growth of

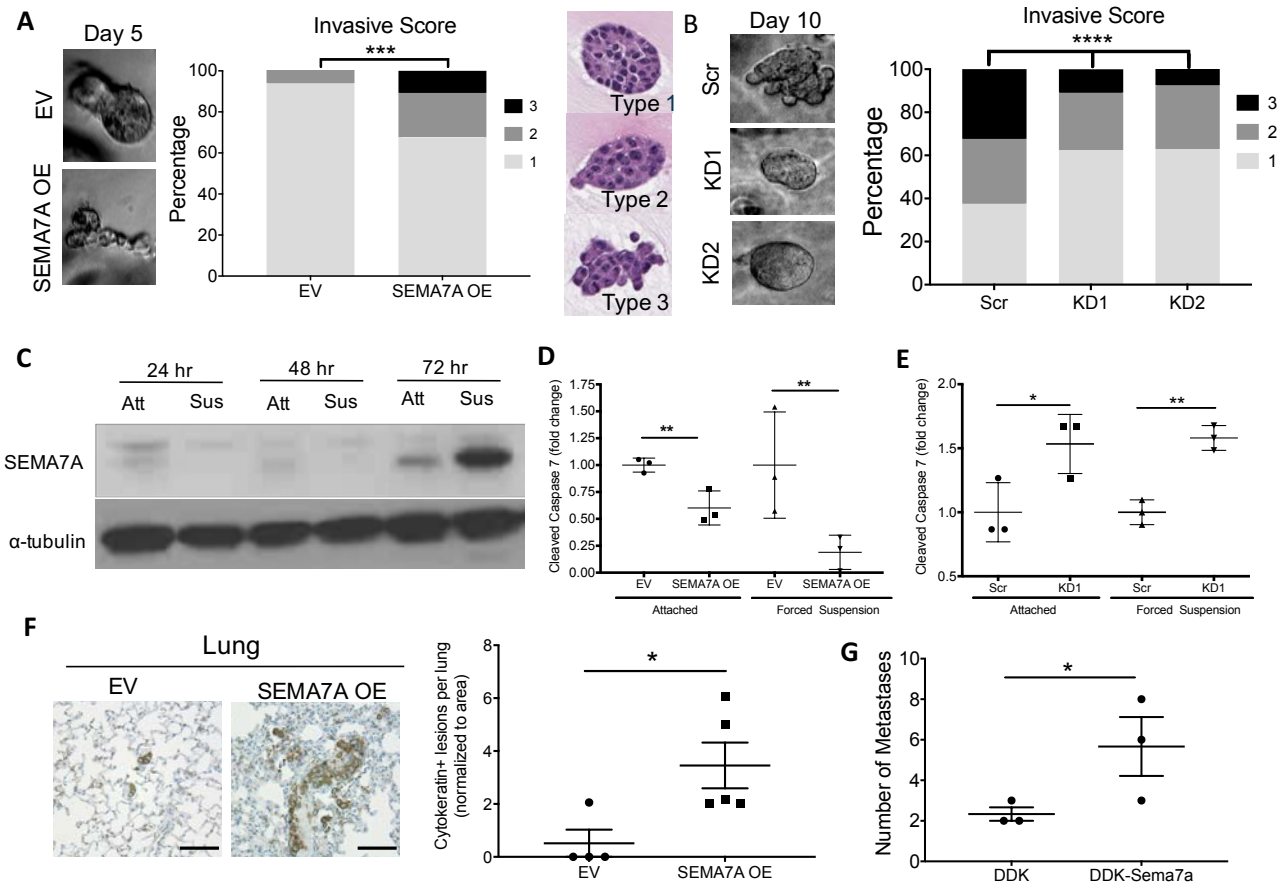

Figure 5: SEMA7A promotes metastatic phenotypes. (A) MCF7 EV and SEMA7A OE cells embedded in Matrigel plus 20% collagen scored for invasive phenotypes after 5 days in 3D culture. Type 1 represents non-invasive organoids, type 2 represents moderately invasive organoids, and type 3 represents highly invasive organoids. Representative live images (left), quantification (middle), and H&Es of typing system right) are shown. **(B)** MCF7 Scr, KD1, or KD2 cells embedded in Matrigel plus 20% collagen scored for invasive phenotypes after 10 days in 3D culture. Representative live images (left) and quantification (right) are shown. **(C)** Immunoblot for SEMA7A in wildtype MCF7 cells cultured in normal attached (Att) or forced suspension (Sus) conditions over a timecourse. **(D)** Cleaved caspase 7 activity in MCF7 EV and SEMA7A OE (n = 3) or **(E)** Scr and KD1 cells grown in attached or forced suspension cultures for 48 hours (n = 3). Data normalized to EV or Scr controls in attached conditions. **(F)** Representative IHC for pan-cytokeratin (left) and quantification (right) in lungs from mice with MCF7 EV or SEMA7A OE tumors (n=4-6 per group). Scale bars 50 µm. **(G)** Number of detectable lung metastases per lung lobe from mice that received tail vein injections of E0771 DDK or DDK-Sema7a cells (n=3). *$p < 0.05$, **$p<0.01$, ***$p < 0.005$, ****$p < 0.001$ by unpaired two-sided t-test (A, F, G) or one-way ANOVA with Tukey's multiple comparison test (B,D, E)

SEMA7A expressing cells. As expected, when treated with the CDK 4/6 inhibitor palbociclib, empty vector control MCF7 cells exhibited a significant reduction of growth and clonogenicity *in vitro* compared to vehicle treatment; however, growth of SEMA7A OE cells was not significantly affected (Figure 6A-B and SFigure 5A). We therefore sought to identify therapies that would specifically target SEMA7A-expressing tumors; using an Oncomine query to identify potential compounds and targetable pathways, we identified 6 compounds predicted to elicit sensitivity of SEMA7A+ tumors, all of which target proteins in either the MAPK or PI3K/AKT signaling pathways (STable 4). Notably, published studies describe these pathways as downstream signaling mediators of SEMA7A activity (2, 5, 8). Thus, we tested whether growth of SEMA7A OE cells is inhibited by commercially available small molecule inhibitors that target Src and Akt, SU6656 and LY294002, respectively. We observed significantly decreased growth of SEMA7A OE cells with both inhibitors (SFigure 5B-C). However, as these compounds are not clinically available, we sought to identify a compound that would inhibit the downstream pro-survival function induced by these pathways. Venetoclax is an orally available inhibitor of the anti-apoptotic BCL2 protein that has recently demonstrated synergy in combination therapy approaches in patients with ER+ breast cancer (23). We therefore tested the sensitivity of SEMA7A OE cells to venetoclax. Proliferation and clonogenic outgrowth of SEMA7A OE cells was decreased with venetoclax treatment as compared to vehicle treatment and to EV control cells treated with venetoclax (Figure 6C-D and SFigure 5D). Finally, to test whether SEMA7A OE tumors respond to venetoclax treatment *in* vivo, we implanted MCF7 control or SEMA7A OE cells into NCG mice. Tumors were allowed to grow to an average of 200mm^3, when we then initiated fulvestrant treatment. As expected, the SEMA7A OE tumors continued to grow, while tumor growth plateaued in the control group. After separation of the curves was observed, we administered venetoclax treatment in combination with fulvestrant. We observed a dramatic decrease in tumor growth rate in the *SEMA7A* OE, which was not observed in the control group (Figure 6E-F and SFigure E). Thus, our results suggest that venetoclax may be an viable treatment option for inhibiting growth, recurrence, and metastasis of SEMA7A-expressing ER+ breast cancers, which are likely to be resistant to endocrine therapies.

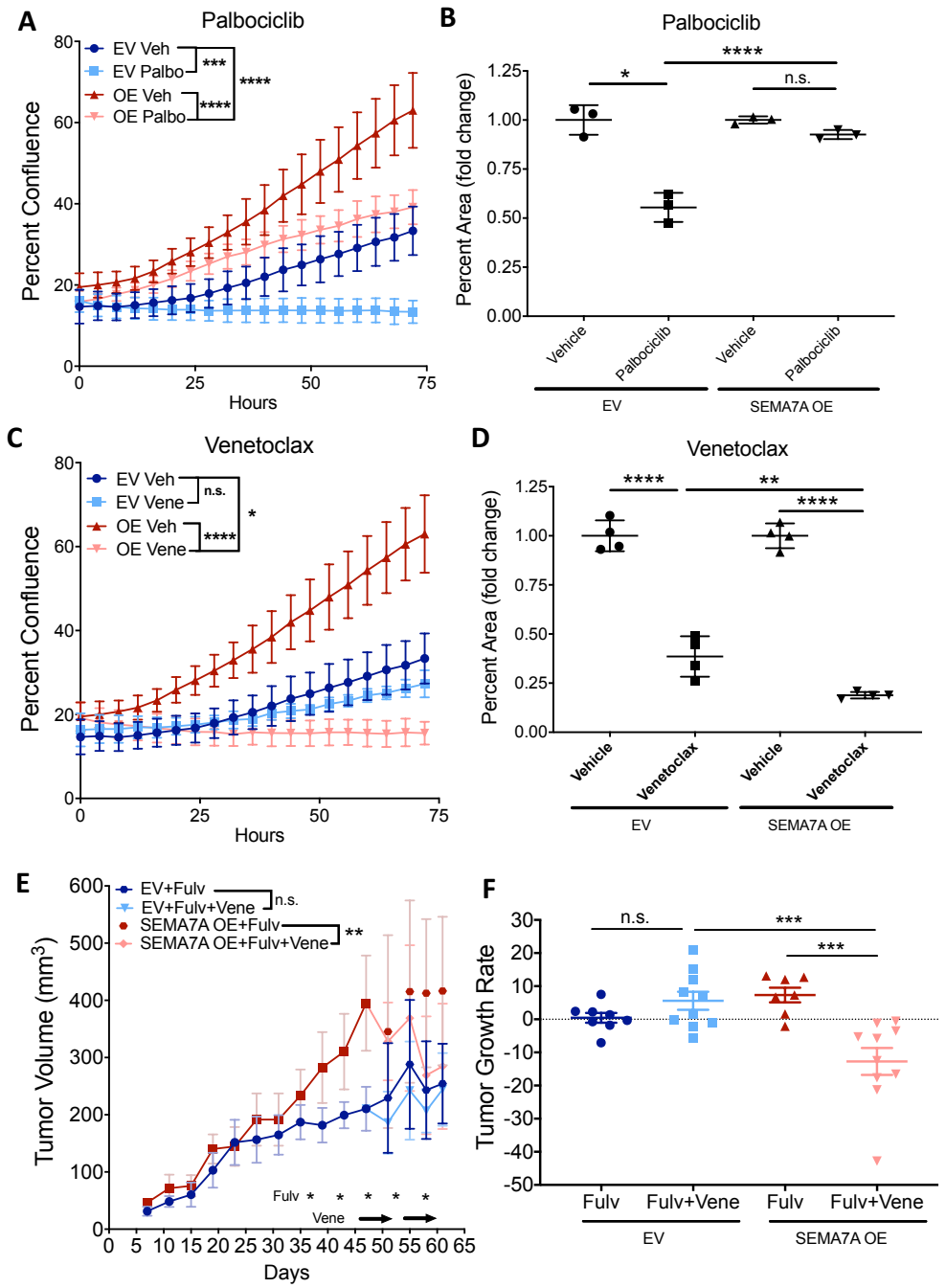

Figure 6: Therapeutic vulnerability of SEMA7A-expressing tumors. (A) Confluence and **(B)** clonogenic growth of MCF7 EV or SEMA7A OE cells treated with 2.5μM palbociclib. Clonogenic data are normalized to EV vehicle controls (n = 3). **(C)** Confluence and **(B)** clonogenic growth of MCF7 EV or SEMA7A OE cells treated with 300nM venetoclax. Clonogenic data are normalized to EV vehicle controls (n = 3). **(E)** Tumor volumes of MCF7 EV and SEMA7A OE tumors in hosts treated with fulvestrant and venetoclax or vehicle (n = 4/group). **(F)** Tumor growth rate (average change in tumor volume per day) of EV or SEMA7A OE MCF7 tumors on fulvestrant and venetoclax or vehicle treatment. $p < 0.05$, $^{**}p<0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$ by one-way ANOVA with Tukey's multiple comparison test (B,D,F) or repeated measures one-way ANOVA with Tukey's multiple comparison test (A,C,E)

DISCUSSION

Determining whether patients with ER+ breast cancer will or will not respond to endocrine therapy and identifying novel treatment options for patients with endocrine resistant ER+ breast cancer is of the utmost importance, as patients with ER+ disease comprise the largest percentage of breast cancer patients and the largest number of breast cancer-related deaths. The semaphorin family of proteins have multiple, and variable, described roles in cancer, with some demonstrating tumor-suppressive functions and others demonstrating tumor-promotional roles (24). The data presented in this study contribute to an emerging body of evidence describing a role for SEMA7A in tumor progression. Our lab and others have recently begun to characterize SEMA7A as a mediator of breast/mammary, melanoma, and lung cancer (9-14). In this report, we describe a novel role for SEMA7A in ER+ breast cancer recurrence and metastasis, where SEMA7A expression confers resistance to anti-endocrine therapy and predicts for poor response in patients with ER+ disease. In previously published data, we showed that SEMA7A promotes numerous tumor-promotional phenotypes in preclinical models, including tumor cell growth, motility, invasion, and cell survival (9, 15). We also previously reported that SEMA7A induces macrophage-mediated remodeling of lymphatic vasculature, as well as tumor cell dissemination via the lymphatics, and others have demonstrated SEMA7A-dependent angiogenesis in ER- breast cancer models (10, 11).

Motility and invasion are known hallmarks of metastatic cells (22). However, as cells venture through the metastatic cascade, they must also adapt to many different microenvironments such as certain hostile conditions, including anchorage independent conditions during travel through the vasculature or lymphatics. We now show that SEMA7A expression promotes anoikis resistance and consequent anchorage-independent survival and we extend our observations to show that SEMA7A overexpression can promote spontaneous and experimental lung metastasis. Additionally, we suggest that the pleiotropic effects promoted by SEMA7A may give SEMA7A-expressing tumors a selective advantage under the stress of endocrine therapies, which results in long-term hormonal depravation intended to halt tumor cell growth and induce cell death. Our results support that SEMA7A expression allows tumor cells to bypass reliance on ER, rendering them reliant instead upon SEMA7A mediated cell survival. This is further evidenced by our observations that SEMA7A-expressing tumors

downregulate expression of ER—a mechanism that warrants further investigation. Furthermore, published studies report that SEMA4C promotes MCF7 tumor growth, viability, metastasis, and renders ER+ cells non-responsive to tamoxifen via downregulation of ER (25), suggesting that this mechanism may extend to other multiple semaphorin family members.

Metastatic disease in patients with ER+ breast cancer frequently exhibits endocrine resistance, and many metastases downregulate ER or express mutant forms of the *ESR1* gene. Although the prevalence of ER loss has been debated, a recent meta-analysis reveals approximately 23% of ER+ tumors have corresponding ER- metastases (21). Further, tumors with low expression of ER (1-10% positive) are reported to behave more like ER- tumors, and patients with ER low tumors have significantly lower disease free survival rates compared to patients with ER high (11-100% positive) tumors (26). These results are consistent with our observations that SEMA7A expressing cells exhibit pro-metastatic phenotypes and are also resistance to therapy and downregulate ER. We suggest that initial expression of SEMA7A in hormone receptor positive disease is promoted through ER and PR signaling, as we demonstrate that expression of SEMA7A can be promoted by hormone receptor signaling through both ER and PR. However, SEMA7A expression does not uniformly decrease in patients treated with hormone-targeting endocrine therapies. Instead, endocrine therapy induces one of two responses: 1) SEMA7A levels decrease, which correlates with a good overall response to endocrine therapy or 2) SEMA7A levels increase, which correlates with a poor response to endocrine therapy. Studies of other semaphorins have also described hormone receptor regulation. In ovarian cancer, pro-proliferative *SEMA4D* is positively regulated by ER (27). Expression of *SEMA3B* and *SEMA3F*, which are both growth-suppressive, in ovarian cancer is positively regulated by E2 and P4 (28). Finally, *SEMA3C* is transcriptionally regulated by the androgen receptor in prostate cancer cells (29). Functionally, SEMA3C promotes growth, migration, and invasion in breast cancer (30), is detectable in metastatic lung cancer cells (31), and promotes survival of gastric cancer and glioma stem cells (32, 33). SEMA3C also has a described role in promoting drug resistance in ovarian cancer (34). Our results suggest that SEMA7A expression predicts for poor outcomes in patients that are hormone receptor positive, particularly in the PR+ subset. Furthermore, since PR is a transcriptional target of ER, our data showing that SEMA7A expression is significantly

correlated with PR expression suggests that SEMA7A is associated with ER and PR activity. The role of PR in breast cancer is controversial and complicated, with data supporting that PR can both agonize and antagonize ER, depending on the context (35), and that PR can modulate ER activity and alter ER-regulated gene expression (36). Activity of other signaling pathways can also affect PR activity; for example, the MAPK/ERK pathway alters phosphorylation of PR and its co-regulators (37). More recent data from Truong et al. demonstrate that divergent PR activity, including activation of distinct pathways promoting or inhibiting disease recurrence, is determined by PR phosphorylation and isoform status (38), neither of which are currently screened for in the clinic. Our data suggest that SEMA7A may be another novel predictor of outcome for patients with PR+ disease, and SEMA7A status may help to better characterize these divergent responses in PR+ breast cancer.

In addition to endocrine therapy resistance, we demonstrate that SEMA7A expression confers resistance to the CDK 4/6 inhibitor palbociclib, which is used in combination with endocrine therapy for first line treatment of metastatic disease or in subsequent lines following disease progression (39). Our data suggest that patients with SEMA7A+ tumors may be at high risk for relapse and poor response to current standard-of-care therapies, and that novel therapeutic strategies could be applied for patients pre-selected by SEMA7A status. Interestingly, Kinehara et al. recently described that SEMA7A promotes resistance to standard-of-care EGFR tyrosine kinase inhibitors in lung cancer through anti-apoptotic β1-integrin-mediated ERK signaling; SEMA7A was thus proposed as a predictive marker for response to TKI therapy and potential therapeutic target for resistant tumors (14). This report, along with our findings, suggests that SEMA7A may confer resistance to multiple targeted therapies in numerous tumor types; therefore, direct targeting and therapeutic vulnerabilities of SEMA7A+ tumors warrant additional investigation in a clinical setting. Moving toward this goal, results presented herein suggest that SEMA7A+ tumors may be targetable via other mechanisms. Specifically, SEMA7A has demonstrated the ability to activate PI3K/AKT and MAPK/ERK signaling (2, 5, 8). We tested if SEMA7A+ tumor cells exhibit sensitivity to selective inhibitors of these pathways and venetoclax, a downstream anti-apoptotic effector targeting BCL2. Our results showing that SEMA7A+ tumors cells exhibit reduced growth and viability after exposure to these therapeutics support that targeting downstream effectors of SEMA7A may prove an

effective strategy for recurrent SEMA7A+ tumors resistant to currently available therapeutic regimes. Previous reports have described increased BCL2 expression in fulvestrant-resistant MCF7 cells, and fulvestrant sensitivity can be restored with a BCL2 inhibitor or BCL2 knockdown (40). Additionally, the first clinical study of the BCL2 inhibitor venetoclax in solid tumors revealed that ER+ metastatic patients received great benefit from this drug in combination with tamoxifen, and a recent report describes a benefit of adding venetoclax to palbociclib in preclinical ER+ models (41). Since venetoclax is clinically available, administered orally, and well-tolerated, we propose that novel clinical trials could test whether patients with ER+SEMA7A+ cancer would benefit from combination treatment in both the adjuvant and metastatic setting. Finally, clinical trials assessing SEMA4C as a biomarker for breast cancer diagnosis and relapse are already underway (ClinicalTrials.gov identifiers: NCT03663153 and NCT03662633). Since similar phenotypes are observed in pre-clinical models with SEMA4C and SEMA7A, we propose that SEMA7A may also warrant investigation in the clinic as a predictive marker of ER+ breast cancer recurrence.

ACKNOWLEDGEMENTS

The authors acknowledge the CU Anschutz Cancer Center Protein Production/MoAB/Tissue Culture Shared Resource, the Biorepository Core Facility, and the University of Colorado Cancer Center Support Grant (P30CA046934) for SEMA7A protein production, Incucyte use, and Aperio Software use. Contents are the authors' sole responsibility and do not necessarily represent official NIH views. The authors also thank the following persons for technical support and feedback in the production of this manuscript: Alan Elder, Alexander Stoller, Veronica Wessells, Michelle Borakove, Virginia Borges, Chloe Young, Sarah Tarullo, Annie Pham, and Paris Franz.

Author Contributions:

LSC and TRL conceived and designed the study. LSC performed all *in vivo* studies. LSC and GLW performed all *in vitro* studies. JKR and WWP assisted with methodology development and data interpretation. GLW, WWP, and JKR provided critical reagents. LSC and TRL were responsible for hypothesis development, conceptual design, and data analysis. LSC and TRL wrote the manuscript, and all authors provided critical evaluation and review of the manuscript.

REFERENCES

1. H. Pan, R. Gray, J. Braybrooke, C. Davies, C. Taylor, P. McGale, R. Peto, K. I. Pritchard, J. Bergh, M. Dowsett, D. F. Hayes and Ebctcg. 20-Year Risks of Breast-Cancer Recurrence after Stopping Endocrine Therapy at 5 Years. N Engl J Med. 2017;377:1836-46.
2. R. J. Pasterkamp, J. J. Peschon, M. K. Spriggs and A. L. Kolodkin. Semaphorin 7A promotes axon outgrowth through integrins and MAPKs. Nature. 2003;424:398-405.
3. T. Mine, K. Harada, T. Matsumoto, H. Yamana, K. Shirouzu, K. Itoh and A. Yamada. CDw108 expression during T-cell development. Tissue Antigens. 2000;55:429-36.
4. M. Zhang, L. Wang, M. Dong, Z. Li and F. Jin. Endothelial Semaphorin 7A promotes inflammation in seawater aspiration-induced acute lung injury. Int J Mol Sci. 2014;15:19650-61.
5. K. Suzuki, T. Okuno, M. Yamamoto, R. J. Pasterkamp, N. Takegahara, H. Takamatsu, T. Kitao, J. Takagi, P. D. Rennert, A. L. Kolodkin, A. Kumanogoh and H. Kikutani. Semaphorin 7A initiates T-cell-mediated inflammatory responses through alpha1beta1 integrin. Nature. 2007;446:680-4.
6. J. C. Morote-Garcia, D. Napiwotzky, D. Kohler and P. Rosenberger. Endothelial Semaphorin 7A promotes neutrophil migration during hypoxia. Proc Natl Acad Sci U S A. 2012;109:14146-51.
7. J. Parkash, A. Messina, F. Langlet, I. Cimino, A. Loyens, D. Mazur, S. Gallet, E. Balland, S. A. Malone, F. Pralong, G. Cagnoni, R. Schellino, S. De Marchis, M. Mazzone, R. J. Pasterkamp, L. Tamagnone, V. Prevot and P. Giacobini. Semaphorin7A regulates neuroglial plasticity in the adult hypothalamic median eminence. Nat Commun. 2015;6:6385.
8. H.-R. Kang, C. G. Lee, R. J. Homer and J. a. Elias. Semaphorin 7A plays a critical role in TGF-beta1-induced pulmonary fibrosis. The Journal of experimental medicine. 2007;204:1083-93.
9. S. A. Black, A. C. Nelson, N. J. Gurule, B. W. Futscher and T. R. Lyons. Semaphorin 7a exerts pleiotropic effects to promote breast tumor progression. Oncogene. 2016;35:5170-8.
10. A. M. Elder, B. A. J. Tamburini, L. S. Crump, S. A. Black, V. M. Wessells, P. J. Schedin, V. F. Borges and T. R. Lyons. Semaphorin 7A Promotes Macrophage-Mediated Lymphatic Remodeling during Postpartum Mammary Gland Involution and in Breast Cancer. Cancer Res. 2018;78:6473-85.
11. R. Garcia-Areas, S. Libreros, S. Amat, P. Keating, R. Carrio, P. Robinson, C. Blieden and V. Iragavarapu-Charyulu. Semaphorin7A promotes tumor growth and exerts a pro-angiogenic effect in macrophages of mammary tumor-bearing mice. Frontiers in physiology. 2014;5:17.
12. B. Ma, E. L. Herzog, C. G. Lee, X. Peng, C. M. Lee, X. Chen, S. Rockwell, J. S. Koo, H. Kluger, R. S. Herbst, M. Sznol and J. A. Elias. Role of chitinase 3-like-1 and semaphorin 7a in pulmonary melanoma metastasis. Cancer Res. 2015;75:487-96.
13. R. Garcia-Areas, S. Libreros, M. Simoes, C. Castro-Silva, N. Gazaniga, S. Amat, J. Jaczewska, P. Keating, K. Schilling, M. Brito, E. P. Wojcikiewicz and V. Iragavarpu-Charyulu. Suppression of tumor-derived Semaphorin 7A and genetic ablation of host-derived Semaphorin 7A impairs tumor progression in a murine model of advanced breast carcinoma. Int J Oncol. 2017;51:1395-404.
14. Y. Kinehara, I. Nagatomo, S. Koyama, D. Ito, S. Nojima, R. Kurebayashi, Y. Nakanishi, Y. Suga, Y. Nishijima-Futami, A. Osa, T. Nakatani, Y. Kato, M. Nishide, Y. Hayama, M. Higashiguchi, O. Morimura, K. Miyake, S. Kang, T. Minami, H. Hirata, K. Iwahori, T. Takimoto, H. Takamatsu, Y. Takeda, N. Hosen, S. Hoshino, Y. Shintani, M. Okumura, T. Kumagai, K. Nishino, F. Imamura, S. I. Nakatsuka, T. Kijima, H. Kida and A. Kumanogoh. Semaphorin 7A promotes EGFR-TKI resistance in EGFR mutant lung adenocarcinoma cells. JCI Insight. 2018;3.
15. S. E. Tarullo, R. C. Hill, K. C. Hansen, F. Behbod, V. F. Borges, A. C. Nelson and T. R. Lyons. Postpartum breast cancer progression is driven by semaphorin 7a-mediated invasion and survival. Oncogene. 2020.

16. T. R. Lyons, J. O'Brien, V. F. Borges, M. W. Conklin, P. J. Keely, K. W. Eliceiri, A. Marusyk, A. C. Tan and P. Schedin. Postpartum mammary gland involution drives progression of ductal carcinoma in situ through collagen and COX-2. Nat Med. 2011;17:1109-15.
17. J. O'Brien, T. Lyons, J. Monks, M. S. Lucia, R. S. Wilson, L. Hines, Y. G. Man, V. Borges and P. Schedin. Alternatively activated macrophages and collagen remodeling characterize the postpartum involuting mammary gland across species. Am J Pathol. 2010;176:1241-55.
18. A. Nagy, A. Lanczky, O. Menyhart and B. Gyorffy. Validation of miRNA prognostic power in hepatocellular carcinoma using expression data of independent datasets. Sci Rep. 2018;8:9227.
19. A. K. Turnbull, L. M. Arthur, L. Renshaw, A. A. Larionov, C. Kay, A. K. Dunbier, J. S. Thomas, M. Dowsett, A. H. Sims and J. M. Dixon. Accurate Prediction and Validation of Response to Endocrine Therapy in Breast Cancer. J Clin Oncol. 2015;33:2270-8.
20. L. Kreft, A. Soete, P. Hulpiau, A. Botzki, Y. Saeys and P. De Bleser. ConTra v3: a tool to identify transcription factor binding sites across species, update 2017. Nucleic Acids Res. 2017;45:W490-W4.
21. W. Schrijver, K. P. M. Suijkerbuijk, C. H. van Gils, E. van der Wall, C. B. Moelans and P. J. van Diest. Receptor Conversion in Distant Breast Cancer Metastases: A Systematic Review and Meta-analysis. J Natl Cancer Inst. 2018;110:568-80.
22. D. R. Welch and D. R. Hurst. Defining the Hallmarks of Metastasis. Cancer Res. 2019.
23. S. W. Lok, J. R. Whittle, F. Vaillant, C. E. Teh, L. L. Lo, A. N. Policheni, A. R. T. Bergin, J. Desai, S. Ftouni, L. C. Gandolfo, D. Liew, H. K. Liu, G. B. Mann, K. Moodie, A. Murugasu, B. Pal, A. W. Roberts, M. A. Rosenthal, K. Shackleton, M. J. Silva, Z. R. Siow, G. K. Smyth, L. Taylor, A. Travers, B. Yeo, M. M. Yeung, A. Z. Bujak, S. J. Dawson, D. H. D. Gray, J. E. Visvader and G. J. Lindeman. A Phase Ib Dose-Escalation and Expansion Study of the BCL2 Inhibitor Venetoclax Combined with Tamoxifen in ER and BCL2-Positive Metastatic Breast Cancer. Cancer Discov. 2019;9:354-69.
24. G. Neufeld, Y. Mumblat, T. Smolkin, S. Toledano, I. Nir-Zvi, K. Ziv and O. Kessler. The role of the semaphorins in cancer. Cell Adh Migr. 2016;10:652-74.
25. S. Gurrapu, E. Pupo, G. Franzolin, L. Lanzetti and L. Tamagnone. Sema4C/PlexinB2 signaling controls breast cancer cell growth, hormonal dependence and tumorigenic potential. Cell Death Differ. 2018;25:1259-75.
26. J. S. Prabhu, A. Korlimarla, K. Desai, A. Alexander, R. Raghavan, C. Anupama, N. Dendukuri, S. Manjunath, M. Correa, N. Raman, A. Kalamdani, M. Prasad, K. S. Gopinath, B. S. Srinath and T. S. Sridhar. A Majority of Low (1-10%) ER Positive Breast Cancers Behave Like Hormone Receptor Negative Tumors. J Cancer. 2014;5:156-65.
27. Y. Liu, Y. Hou, L. Ma, C. Sun, J. Pan, Y. Yang, H. Zhou and J. Zhang. Regulation of semaphorin 4D expression and cell proliferation of ovarian cancer by ERalpha and ERbeta. Braz J Med Biol Res. 2017;50:e6057.
28. D. Joseph, S. M. Ho and V. Syed. Hormonal regulation and distinct functions of semaphorin-3B and semaphorin-3F in ovarian cancer. Mol Cancer Ther. 2010;9:499-509.
29. K. J. Tam, K. Dalal, M. Hsing, C. W. Cheng, S. Khosravi, P. Yenki, C. Tse, J. W. Peacock, A. Sharma, Y. T. Chiang, Y. Wang, A. Cherkasov, P. S. Rennie, M. E. Gleave and C. J. Ong. Androgen receptor transcriptionally regulates semaphorin 3C in a GATA2-dependent manner. Oncotarget. 2017;8:9617-33.
30. C. Esselens, J. Malapeira, N. Colome, C. Casal, J. C. Rodriguez-Manzaneque, F. Canals and J. Arribas. The cleavage of semaphorin 3C induced by ADAMTS1 promotes cell migration. J Biol Chem. 2010;285:2463-73.
31. M. Martin-Satue and J. Blanco. Identification of semaphorin E gene expression in metastatic human lung adenocarcinoma cells by mRNA differential display. J Surg Oncol. 1999;72:18-23.
32. H. Miyato, N. H. Tsuno and J. Kitayama. Semaphorin 3C is involved in the progression of gastric cancer. Cancer Sci. 2012;103:1961-6.
33. J. Man, J. Shoemake, W. Zhou, X. Fang, Q. Wu, A. Rizzo, R. Prayson, S. Bao, J. N. Rich and J. S. Yu. Sema3C promotes the survival and tumorigenicity of glioma stem cells through Rac1 activation. Cell Rep. 2014;9:1812-26.

34. T. Yamada, R. Endo, M. Gotoh and S. Hirohashi. Identification of semaphorin E as a non-MDR drug resistance gene of human cancers. Proc Natl Acad Sci U S A. 1997;94:14713-8.
35. H. Singhal, M. E. Greene, G. Tarulli, A. L. Zarnke, R. J. Bourgo, M. Laine, Y. F. Chang, S. Ma, A. G. Dembo, G. V. Raj, T. E. Hickey, W. D. Tilley and G. L. Greene. Genomic agonism and phenotypic antagonism between estrogen and progesterone receptors in breast cancer. Sci Adv. 2016;2:e1501924.
36. H. Mohammed, I. A. Russell, R. Stark, O. M. Rueda, T. E. Hickey, G. A. Tarulli, A. A. Serandour, S. N. Birrell, A. Bruna, A. Saadi, S. Menon, J. Hadfield, M. Pugh, G. V. Raj, G. D. Brown, C. D'Santos, J. L. Robinson, G. Silva, R. Launchbury, C. M. Perou, J. Stingl, C. Caldas, W. D. Tilley and J. S. Carroll. Progesterone receptor modulates ERalpha action in breast cancer. Nature. 2015;523:313-7.
37. L. S. Trevino, W. E. Bingman, 3rd, D. P. Edwards and W. Nl. The requirement for p42/p44 MAPK activity in progesterone receptor-mediated gene regulation is target gene-specific. Steroids. 2013;78:542-7.
38. T. H. Truong, A. R. Dwyer, C. H. Diep, H. Hu, K. M. Hagen and C. A. Lange. Phosphorylated Progesterone Receptor Isoforms Mediate Opposing Stem Cell and Proliferative Breast Cancer Cell Fates. Endocrinology. 2019;160:430-46.
39. J. A. Beaver, L. Amiri-Kordestani, R. Charlab, W. Chen, T. Palmby, A. Tilley, J. F. Zirkelbach, J. Yu, Q. Liu, L. Zhao, J. Crich, X. H. Chen, M. Hughes, E. Bloomquist, S. Tang, R. Sridhara, P. G. Kluetz, G. Kim, A. Ibrahim, R. Pazdur and P. Cortazar. FDA Approval: Palbociclib for the Treatment of Postmenopausal Patients with Estrogen Receptor-Positive, HER2-Negative Metastatic Breast Cancer. Clin Cancer Res. 2015;21:4760-6.
40. A. C. Crawford, R. B. Riggins, A. N. Shajahan, A. Zwart and R. Clarke. Co-inhibition of BCL-W and BCL2 restores antiestrogen sensitivity through BECN1 and promotes an autophagy-associated necrosis. PLoS One. 2010;5:e8604.
41. J. R. Whittle, F. Vaillant, E. Surgenor, A. N. Policheni, G. Giner, B. D. Capaldo, H. R. Chen, H. K. Liu, J. F. Dekkers, N. Sachs, H. Clevers, A. Fellowes, T. Green, H. Xu, S. B. Fox, M. J. Herold, G. K. Smyth, D. H. D. Gray, J. E. Visvader and G. J. Lindeman. Dual targeting of CDK4/6 and BCL2 pathways augments tumor response in estrogen receptor positive breast cancer. Clin Cancer Res. 2020.