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

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ABSTRACT

Breast cancer is a leading cause of cancer-associated death in women in the United States, and approximately 70% of all cases are estrogen receptor positive (ER+). While effective ER-targeting endocrine therapy has substantially progressed the successful treatment of primary ER+ tumors, approximately 20% of tumors recur, typically as metastases. Recurrent ER+ tumors consistently develop resistance to endocrine therapies, making them difficult to treat, and contributing to patient death. Therefore, novel molecular targets are needed to treat recurrent ER+ breast cancer. Here, we describe semaphorin 7a (SEMA7A) as a driver of tumor growth, recurrence, and metastasis in

ER+ breast cancer. SEMA7A is a signaling protein that is overexpressed in ER+ breast cancer, where it confers significantly decreased patient survival rates for patients on endocrine therapy. We show that *SEMA7A* is hormonally regulated in ER+ breast cancer; yet, *SEMA7A* expression does not uniformly decrease in patients treated with endocrine therapies. Instead, endocrine therapy induces one of two outcomes: either 1) decreases SEMA7A and correlates with a good clinical response or 2) increases SEMA7A and correlates with a poor clinical response. We overexpressed *SEMA7A* in ER+ cell lines and performed in vitro growth assays in the presence of the ER-degrader fulvestrant. We also injected these cells into mammary fat pads of NSG mice, and then treated the animals with fulvestrant. Tumors were measured every three days, then harvested for immunohistochemical analysis. *SEMA7A* overexpression confers resistance to fulvestrant treatment in vitro and in vivo. Mechanistically, we show that SEMA7A suppresses expression of ER and alters the tumor microenvironment in fulvestrant-treated tumors. These data support SEMA7A as a novel driver of endocrine resistance in ER+ breast cancer. Finally, we identify therapeutic vulnerability of ER+SEMA7A+ tumors and propose that SEMA7A warrants additional investigation in a clinical setting.

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 therapies, including aromatase inhibitors and selective estrogen receptor modulators (e.g., tamoxifen) or degraders (e.g., fulvestrant), has facilitated successful treatment of ER+ primary tumors. Yet, 1 in 5 women with ER+ breast cancer will experience disease recurrence, frequently at distant metastatic sites, such as lung, liver, or brain. These recurrent ER+ breast tumors uniformly develop resistance to anti-estrogen-based therapies, rendering most therapeutic interventions in the clinical setting ineffective for treating metastatic tumor growth. Therefore, novel molecular targets are needed to 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[1-5]. In adults, SEMA7A is expressed in the brain, bone marrow, lung, and skin, but is minimally expressed in 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)[6]. In pulmonary fibrosis models, *SEMA7A* is regulated by TGF-β[7]. In its normal physiological role, SEMA7A binds to β1-integrin to activate downstream signaling cascades, including the MAPK and PI3K/AKT pathways[1,4,7]. Our group and others have described tumor-promotional roles for SEMA7A in breast cancer, 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, metastatic spread[8-11].

Studies to date have investigated the role of SEMA7A in breast cancer using pre-clinical models of triple negative breast cancer, which lack expression of ER (ER-), PR (PR-), and amplification of HER2 (HER2-). Yet, our previous genomics analysis in patient datasets demonstrated that *SEMA7A* drives poor prognosis in both ER- and ER+ patients and that *SEMA7A* expression levels were highest in luminal A (generally ER+ and/or PR+ and HER2-) breast cancers[8]. Thus, understanding the role of SEMA7A in ER+ and/or PR+ breast cancers is important for understanding its role in clinical progression. Our results presented herein demonstrate that *SEMA7A* mRNA expression is highest in patients with ER+ breast cancer and that SEMA7A induces pleiotropic effects in ER+ breast cancer cells. We describe a novel relationship between steroid hormone receptor action and expression of SEMA7A in breast cancer. Additionally, we report for the first time that expression of SEMA7A confers resistance to endocrine therapy and to CDK 4/6 inhibitors in ER+ pre-clinical models. Finally, using these same pre-clinical models, we identify therapeutic vulnerabilities of SEMA7A-expressing tumors, including therapies that target the MAPK and PI3K/AKT pathways. 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

T47D and BT474 cells were obtained from G. Borges and M. Borakove (University of Colorado Anschutz Medical Campus). E0771 cells were purchased from CH3 Biosystems (Amherst, NY). All cell lines were validated by the Molecular Biology Core at the University of Colorado Anschutz Medical Campus using a Short Tandem Repeat STR analysis. 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 20.

Transfections

For knockdown studies, SEMA7A-targeting shRNA plasmids (shSEMA7A) or a scrambled control (shCtrl; SA Biosciences, Frederik, MD, and the Functional Genomics Facility at the University of Colorado Anschutz Medical Campus) were transfected using X-tremeGENE transfection reagent (Millipore Sigma, St. Louis, MO) as described by the manufacturer. SEMA7A overexpression was achieved using a SEMA7A-Fc plasmid, which was a generous gift from R. Medzhitov (Yale University, New Haven, CT). A pcDNA3.1 empty vector control plasmid was obtained from H. Ford (University of Colorado Anschutz Medical Campus). All other plasmids were obtained from the Functional Genomics Core at the University of Colorado Anschutz Medical Campus. Knockdown and overexpression were regularly 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). 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). 1μg of DNA was added to GeneJuice Transfection Reagent/serum-free medium mixture. Cells were harvested 2 days after transfection using Reporter Lysis Buffer (Promega, Madison, WI). Luciferase activity and total protein were measured, and luciferase activity was normalized to total protein.

Drug Treatments

Fulvestrant, mifepristone, and 17-β estradiol were purchased from Millipore Sigma (St. Louis, MO). Tamoxifen, palbociclib, and SU6656 were acquired from MedChemExpress (Monmouth Junction, NJ) Progesterone was a gift from C. Sartorius (University of Colorado Anschutz Medical Campus). Venetoclax was a gift from B. Stevens and C. Jordan (University of Colorado Anschutz Medical Campus). Cells were cultured in phenol-free medium containing 5-10% charcoal dextran treated FBS (Thermo Scientific, Waltham, MA, USA) for 3 days as required before beginning treatment.

Growth, Invasion, and Cell Death Assays

100,000 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 cells were seeded per well of a 6-well dish and assessed 14 days later for clonogenic assays. 3D matrigel and collagen cultures were performed as previously described[8,12]. Cleaved caspase 3/7 was measured using a Caspase Glo assay (Promega, Madison, WI) as described by the manufacturer.

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). The conditions were as follows: 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). 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 in RIPA buffer with 50mM sodium fluoride, 100mM sodium vanadate, and 1X protease inhibitor cocktail (cOmplete Protease Inhibitor, Sigma Aldrich, St. Louis, MO), 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 minimally and uniformly to 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 days, and tumor volume was calculated as (L x L 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 s.c. every 4 days. Tumors were harvested at designated study endpoints. Mammary glands with intact tumor were formalin fixed and paraffin embedded for immunohistochemical analysis.

For metastatic 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. Lung, liver, and spleen 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[13]. Antibody information is provided in Supplementary Table 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 Meier Plotter[14] 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 1881 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.[15]. Data were stratified by clinical response (responders vs non-responders) and graphed by timepoint. Linear regression was performed for each group.

The Oncomine Platform (https://www.oncomine.org) was used to query *SEMA7A* in breast cancer. 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 screen.

Flow Cytometry

Post-drug treatment cell viability was determined with Zombie Aqua Fixable Viability Dye (BioLegend, San Diego, CA) according to manufacturer's instructions. Flow cytometry was performed by the University of Colorado Flow Cytometry Core.

Statistical analysis

Statistical evaluations for experiments using cell lines and mice were performed in triplicate unless otherwise noted. Data are expressed as means ± SEM for animal studies and ± SD for *in vitro* studies. A 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 was used to compare groups of 3 or more. 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 in a panel of 51 human breast cancer cell lines using the Gene Expression-based Outcome for Breast Cancer Online (GOBO) tool. We observed that 67% of luminal subtype cell lines exhibit increased *SEMA7A* expression versus 36-50% of the basal subtypes (Figure 1A). Average expression was higher when ER+ cell lines were separated from TNBC and when luminal (which are typically ER+) cell lines were compared to basal subtype (which are typically ER-; Figure 1B). Using the GOBO tool, we also observed that *SEMA7A* mRNA expression was increased in ER+ patient tumors (Figure 1C). We queried SEMA7A on the Oncomine platform and found that SEMA7A was overexpressed or had a higher copy number rank in ER+ or PR+ disease in 7 additional datasets (Table 1A). We also observed that SEMA7A was highest in stage III and IV, grade 2 and 3, and lymph node positive (LN+) disease in multiple datasets (Table 1B). We next analyzed the GOBO dataset for ER+ patient outcomes based on SEMA7A status, and observed that patients with high SEMA7A expression had decreased distant metastasis free survival and overall survival (Figure 1D and SFigure 1). Additionally, 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 (Figure1E). In fact, the hazard ratio for ER+PR+(HER2+/-)SEMA7A+LN- patients was the highest, suggesting that current indicators of prognosis for ER+ breast cancer patients may not adequately predict for relapse in ER+SEMA7A+ patients.

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

Since regulation of SEMA7A by PR was previously reported in the hypothalamus[6] and PR expression can be regulated by ER mediated transcription[16], we sought to determine whether SEMA7A expression in breast cancer cells was promoted by liganded ER and/or PR. We first performed an *in silico* analysis of the SEMA7A promoter using the ConTra (conserved transcription factor binding site) web server[17]. We identified two highly conserved estrogen response elements (ERE) and one progesterone response element half site (Figure 2A). We treated MCF7 cells (which are ER+ and can be induced to express PR with estrogen treatment) after being grown in hormone depleted conditions with estradiol (E2) and/or progesterone (P4). SEMA7A mRNA and protein were induced slightly by E2, but much more by E2+P4 (SFigure 2 and Figure 2B). We inhibited ER with the ER degrader fulvestrant and observed a robust reduction in SEMA7A expression (Figure 2C). To confirm specificity, we utilized a *SEMA7A* luciferase promoter construct to measure activity following E2 treatment and observed a significant increase in luminescence, supporting regulation of *SEMA7A* by E2 (Figure 2D). Since SEMA7A levels were highest in our dual treated (E2+P4) (Figure 2B) we treated T47D cells, which are ER+ and have constitutive expression of PR protein, with P4 alone and with an inhibitor of PR, mifepristone (Figure 2E), and with the combination of E2 and P4 with and without mifepristone and fulvestrant (Figure 2F) to determine if P4 alone stimulated expression of SEMA7A. Further, we observed that ER inhibition was not sufficient to block SEMA7A expression when P4 and PR are present, suggesting that PR can upregulate *SEMA7A* independently of ER. To determine whether new protein synthesis, presumably of PR, 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 and found that CHX treatment blocked E2/P4 mediated SEMA7A expression (Figure 2G).

To determine whether *SEMA7A* gene expression correlates with the genes encoding for ER or PR—*ESR1* and *PGR*—in breast cancers, we examined the METABRIC dataset, which contains gene expression data from over 2,000 patients. We found that *SEMA7A* did not significantly correlate with *ESR1* expression ($p = 0.6043$), but did significantly correlate with *PGR* expression ($p = 0.0253$; data not shown). 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 with due to the n = 22 patients (Figure 2H).

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

To determine if SEMA7A promotes ER+ breast cancer via increased tumor growth, we generated two independent shRNA mediated knockdown (KD) variants of *SEMA7A* in MCF7 cells (Figure 3A). Using an *in vitro* growth assay, we observed that *SEMA7A* KD cells were less confluent over a 72 hour time course (Figure 3B). Similarly, *SEMA7A* KD cells also exhibited decreased colony formation in a clonogenic assay (Figure 3C). We also assessed proliferation via BrdU incorporation in a 3D organoid system and found that SEMA7A KD organoids had significantly reduced proliferation (Figure 3D). Conversely, when we overexpressed SEMA7A (SEMA7A OE; Figure 3E), we observed increased cell growth in two-dimensional cultures (Figure 3F), clonogenic assays (Figure 3G), and 3D cultures (Figure 3H). It is worth noting that SEMA7A OE organoids were harvested 5 days prior to SEMA7A KD organoids to account for altered growth rates between conditions, likely accounting for the observed differences between control groups.

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 and published data suggesting that SEMA4C renders ER+ cells non-responsive to tamoxifen *in vitro*[18], we sought to determine if SEMA7A confers resistance to ER targeting therapy. We first assessed patient survival by Kaplan Meier analysis of patients who received up to 5 years of treatment with tamoxifen. In this selected cohort, high *SEMA7A* expression predicted for increased relapse (Figure 4A). Additionally, in independent analyses of publicly available gene expression data from patients treated with aromatase inhibitors (GSE59515 and GSE20181) where sequencing data is available from a pre-treatment biopsy, at 2 weeks of treatment, and at 3 months of treatment, *SEMA7A* expression increased in patients who did not respond (Figure 4B and SFigure 3).

As these therapies are designed to target ER+ tumors via deprivation of estrogen dependent signaling, we then measured growth of SEMA7A OE cells in estrogen depleted conditions (SFigure 3) or in the presence of the ER degrader, fulvestrant (Figure 4C), using clonogenic assays. In both conditions, SEMA7A OE cells grew significantly better than control cells, with SEMA7A OE in fulvestrant rescuing growth from ~20% confluent to ~70%. As it has been shown that SEMA4C

promotes estrogen independence through decreased expression of ER[18], we also measured *ESR1* mRNA expression in SEMA7A OE cells and observed a 40% decrease (Figure 4D). Notably, we also observed an 89% decrease in *PGR*. These results suggest that *SEMA7A* expression in ER+ breast cancer cells renders them less reliant on hormone dependent receptor signaling for growth, which may also render them resistant to anti-endocrine therapy. To determine whether SEMA7A+ tumor cells also decrease expression of ER and are resistant to fulvestrant *in vivo*, we orthotopically implanted *SEMA7A* OE cells into estrogen supplemented mice. Surprisingly, *SEMA7A* OE tumors showed no initial growth benefit. However, upon treatment with fulvestrant, the control tumors exhibited significantly slowed growth (slope = 5.3 pre-treatment to 4.4 post-treatment) while the *SEMA7A* OE tumors continued to grow at the same rate (slope = 5.7 pre-treatment to 5.9 post-treatment; Figure 4E). At study completion, tumors were harvested for analysis of ER expression and for markers of tumor growth. ER staining intensity by semi-quantitative IHC was significantly reduced in the *SEMA7A* OE tumors (Figure 4F) and the *SEMA7A* OE tumors also exhibited increased expression of the proliferation marker Ki67 (Figure 4G) and less necrosis (Figure 4H).

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

Interestingly, we also observed a robust increase in collagen deposition in our *SEMA7A* OE tumors by trichrome analysis (Figure 5A). Since collagen fibers are known to promote tumor cell invasion, we utilized a 3D culture model whereby we induce invasion with collagen—a model in which we have previously shown that SEMA7A promotes invasion of ER- tumor cells[8,12]—to assess whether SEMA7A promotes ER+ breast cancer cell invasion. Consistent with our hypothesis, most organoids in the control group remained rounded for greater than 5 days post-seeding with evidence of abundant invasive protrusions appearing between 7-10 days in the control cells, which were rarely observed in the knockdowns (images in Figure 5B). In order to assess for invasive morphology in a quantitative manner, we harvested the organoids, paraffin embedded and sectioned them, and preformed hematoxylin and eosin staining; organoids were individually typed as non-invasive (Type 1), moderately invasive (Type 2), or highly invasive (Type 3) based on morphology. We observed a significant reduction in the percentage of invasive Type 3 organoids from ~30% in the scrambled controls to ~5-10% in the knockdowns at ten days after seeding (Figure 5B). We then examined organoids derived from *SEMA7A* OE cells; interestingly, we began

to observe invasive phenotypes as early as 5 days after seeding, which were only evident in the *SEMA7A* OE cells (Figure 5C). These results suggest that *SEMA7A* KD cells are less invasive overall and that *SEMA7A* OE cells invade more rapidly.

Motility and invasion are known hallmarks of metastatic cells[19]. However, as cells venture down the metastatic cascade, they must also adapt to many different microenvironments. As such, metastatic tumor cells are also highly plastic and can survive in certain hostile conditions that would normally result in cell death, including the non-adherent conditions during travel through the vasculature. This would normally induce death of epithelial derived cells via anoikis. Thus, we utilized forced suspension cultures to mimic the anchorage independence necessary for dissemination in the blood or lymphatic vasculature to determine whether SEMA7A is a part of this adaptive process. Using this system, we found that SEMA7A protein increases after 72 hours in suspension (Figure 5D). Then, to determine if SEMA7A expression required for cell survival in forced suspension conditions, we measured cell death via cleaved caspase 7. Using the *SEMA7A* KD cells, we reveal a ~2-fold increase in cell death in both attached and in forced suspension conditions (Figure 5E). Additionally, *SEMA7A* OE resulted in 50% and 80% decreased cell death in attached and suspended conditions, respectively (Figure 5F), suggesting that SEMA7A may mediate protection from cell death both at the primary site and in circulation during metastasis. To analyze this mechanism *in vivo* using an immunocompetent experimental metastasis assay, and to mimic loss of *ESR1* expression and gain of *SEMA7A* expression, we overexpressed *SEMA7A* in the ER- murine mammary carcinoma cell line E0771 (Figure 5G) and performed tail vein injections in estrogen supplemented mice. We observed that *SEMA7A* OE significantly increased metastatic burden in the lungs (Figure 5H), suggesting that SEMA7A expression promotes cell survival in circulation, and potentially in the lung, which 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-expressing tumor cells exhibit multiple hallmarks of metastatic cells and are resistant to anti-endocrine therapy, we postulated that SEMA7A drives metastasis of ER+ tumors. As CDK 4/6 inhibitors have recently been approved and proven effective for patients with metastatic ER+ breast cancer, we tested whether *SEMA7A* OE tumors

would respond to the CDK 4/6 inhibitor palbociclib. As expected, empty vector control MCF7 cells exhibited a significant reduction of growth and viability *in vitro* compared to vehicle treatment; however, growth of *SEMA7A* OE cells was not significantly affected by CDK4/6 inhibition (Figure 6A). We also assessed viability of controls and SEMA7A OE cells with increasing concentrations of palbociclib. We observed that *SEMA7A* OE cells were more viable than controls at 1μM palbociclib, and S*EMA7A* OE cells required a higher dose to reduce viability to the same level as controls. 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 (Table 1C). Notably, published studies describe these pathways as downstream signaling mediators of SEMA7A activity[1,4,7]. Thus, we tested the sensitivity of *SEMA7A* OE cells to select compounds targeting these pathways. Both AKT and SRC inhibition, with LY294002 and Su6656, respectively, significantly decreased growth and viability of *SEMA7A*-expressing tumor cells compared to vehicle treatment (Figure 6B-C and SFigure 4). *SEMA7A* OE cells demonstrated reduced growth and viability to similar levels as control cells with AKT inhibition, while *SEMA7A* OE cells demonstrated notably lower viability than controls with SRC inhibition. These data suggest that AKT and/or SRC inhibitors may be able to target SEMA7A+ tumors that are resistant to anti-endocrine therapy and CDK4/6 inhibitors. Finally, we tested the sensitivity of *SEMA7A*-expressing cells to the Bcl2 inhibitor venetoclax, which has recently demonstrated synergy in combination therapy approaches in patients with ER+ breast cancer[20]. *SEMA7A* OE and controls cells demonstrated a significant decrease in growth with venetoclax treatment (Figure 6D). *SEMA7A* OE and controls were also less viable in a dose-dependent manner with venetoclax, suggesting another potential therapeutic to inhibit SEMA7A-expressing tumors. Overall, our results support that the described targeted therapy strategies may be important treatment options for inhibiting growth of recurrent, SEMA7A-expressing ER+ breast cancers.

DISCUSSION

Determining which patients with ER+ breast cancer will or will not respond to anti-endocrine therapy and identifying novel treatment options for patients with endocrine resistant ER+ breast cancer is of the utmost importance, since ER+ patients 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[21]. Our lab and others have recently begun to specifically characterize SEMA7A as a mediator of breast/mammary cancers, melanoma, and lung cancer[9-11,22]. In this report, we describe a novel role for SEMA7A in ER+ breast cancer, where SEMA7A expression confers resistance to anti-endocrine therapy and predicts for poor patient response. Our current, and previously published data, demonstrate that SEMA7A promotes numerous tumor-promotional phenotypes in preclinical models, including tumor cell growth, invasion, and cell survival. We now propose that these pleiotropic effects may give SEMA7A-expressing tumors a selective advantage under the stress of anti-endocrine therapies, which result in long-term hormonal depravation designed to halt tumor cell growth and induce cell death. We propose that SEMA7A expression allows the tumors to bypass their reliance on canonical ER signaling, rendering them reliant on SEMA7A signaling to induce similar tumor-promotional programs (see model Figure 6E). This is further evidenced by the ability of SEMA7A-expressing tumors to downregulate expression of ER—a mechanism which warrants further investigation.

In addition to anti-endocrine therapy resistance, we demonstrate that SEMA7A expression confers resistance to the CDK 4/6 inhibitor palbociclib, which was recently approved and has proven successful, for use in combination with anti-endocrine therapy for first line treatment of metastatic disease or in subsequent lines following disease progression[23]. Our data suggest that patients with SEMA7A+ tumors may be at high risk for relapse or a poor response to these current standard-of-care therapies, suggesting that novel therapeutic strategies could be applied for patients pre-selected by SEMA7A status. Further supporting this idea, 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[22]. This report, along with our findings, suggests that SEMA7A may confer resistance to multiple targeted therapies in numerous tumor types; thus, direct targeting and therapeutic vulnerabilities of SEMA7A+ tumors warrant additional investigation. Moving toward this goal, additional results presented herein suggest that SEMA7A+ tumors may be targetable via other

mechanisms. Specifically, SEMA7A has demonstrated ability to activate anti-apoptotic PI3K/AKT and MAPK signaling[1,4,7]; we therefore tested if SEMA7A+ tumor cells exhibit sensitivity to selective inhibitors of these pathways and a downstream anti-apoptotic effector, BCL-2. 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 tumors that are resistant to currently available regimes. Since inhibitors of these pathways are clinically available, we propose that novel clinical trials could test whether patients with recurrent/metastatic ER+SEMA7A+ cancer would benefit from such treatments. Additionally, the first clinical study of the BCL-2 inhibitor venetoclax in solid tumors revealed that ER+ metastatic patients received great benefit from this drug regimen in combination with tamoxifen. This promising trial may prove applicable to recurrent ER+SEMA7A+ tumors, which we report respond well to venetoclax.

The data presented in this study contribute to an emerging body of evidence describing a role for SEMA7A in tumor progression. Interestingly, we demonstrate that expression of SEMA7A can be promoted by hormone receptor signaling through both ER and PR; yet, SEMA7A expression does not uniformly decrease in patients treated with endocrine therapies. Instead, endocrine therapy induces one of two distinct responses: 1) SEMA7A levels decrease, which correlates with a good overall response to anti-endocrine therapy or 2) SEMA7A levels increase, which correlates with a poor response to anti-endocrine therapy. Other semaphorins also have previously described relationships with hormone receptors. In ovarian cancer, SEMA4D is pro-proliferative and its expression is positively regulated by ER[24]. Expression of growth-suppressive SEMA3B and SEMA3F in ovarian cancer is also positively regulated by E2 and P4[25]. Additionally, *SEMA4C* is transcriptionally regulated by the androgen receptor in prostate cancer cells[26]. Our results also suggest that SEMA7A expression predicts for diminished patient outcomes especially based on PR+ expression. The role of PR in breast cancer is controversial and complicated, with data supporting that PR agonists can both agonize and antagonize ER, depending on the specific context[27,28]. More recent data from Truong et al. demonstrate that divergent PR activity is also determined by PR phosphorylation and isoform status[29], none of which are currently screened for in the clinic. Our data suggest that SEMA7A may be another novel predictor of outcome for

patients specifically with PR+ disease, and SEMA7A status may help to better characterize these divergent responses in PR+ breast cancer.

ER+ breast cancer patients with metastases exhibit endocrine resistance and a majority of metastases downregulate ER or gain mutations in the *ESR1* gene. These results are consistent with our data suggesting that SEMA7A-expressing cells exhibit pro-metastatic phenotypes, resistance to therapy, and downregulation of ER. We propose that initial expression of SEMA7A in hormone receptor positive disease is promoted through ER and PR signaling. However, we have shown that SEMA7A itself can produce multiple pro-tumorigenic phenotypes within the tumor cells and the tumor microenvironment (TME), one of which is collagen deposition and induction of tumor cell invasion[8,30]. In studies by our lab utilizing ER- cells, we identified that SEMA7A induces collagen deposition in the TME via signaling to fibroblasts[30] and we have also shown that collagen in the TME induces pro-inflammatory signaling and pro-survival signaling via increased expression of cyclooxygenase-2 expression and activity[12]. Interestingly, COX-2 expression and activity feeds forward to promote additional collagen deposition[12], increased SEMA7A expression[8], as well as SEMA7A dependent deposition of a pro-survival and pro-invasive matrix molecule, fibronectin[30]. Our results presented herein also show that SEMA7A can promotes cell survival in both the presence and the absence of matrix attachment, such as what occurs during dissemination via the circulation. Since SEMA7A can ligand β1-integrin—canonically a receptor for extracellular matrix protein—and SEMA7A expressing cells make their own fibronectin to activate pro-survival integrin signaling, we suggest that SEMA7A and/or fibronectin promote survival in the circulation by mimicking matrix attachment. Thus, we propose that crosstalk between cellular signaling and the TME mediated by SEMA7A results in a bypass of the tumor cell's requirement for ER/PR-mediated upregulation of SEMA7A, which will drive metastasis independent of the long-term estrogen deprivation induced in patients on aromatase inhibitor therapy.

In conclusion, we have shown that SEMA7A expression induces numerous tumor-promotional phenotypes and, importantly, resistance to clinically available anticancer drugs in ER+ breast cancer. Targeting SEMA7A and/or its downstream effectors may be an important step in the inhibition of recurrent, metastatic SEMA7A+ tumors. We also suggest that SEMA7A may be a useful biomarker for the prediction of relapse for patients with ER+ disease who receive endocrine

therapy and/or CDK4/6 inhibitors. Clinical trials assessing SEMA4C as a biomarker for breast cancer diagnosis and relapse are already underway (ClinicalTrials.gov identifiers: NCT03663153 and NCT03662633), and we propose that SEMA7A may also warrant investigation in the clinic as a predictive marker of risk of recurrence in ER+ breast cancer.

ACKNOWLEDGEMENTS

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Figure 1

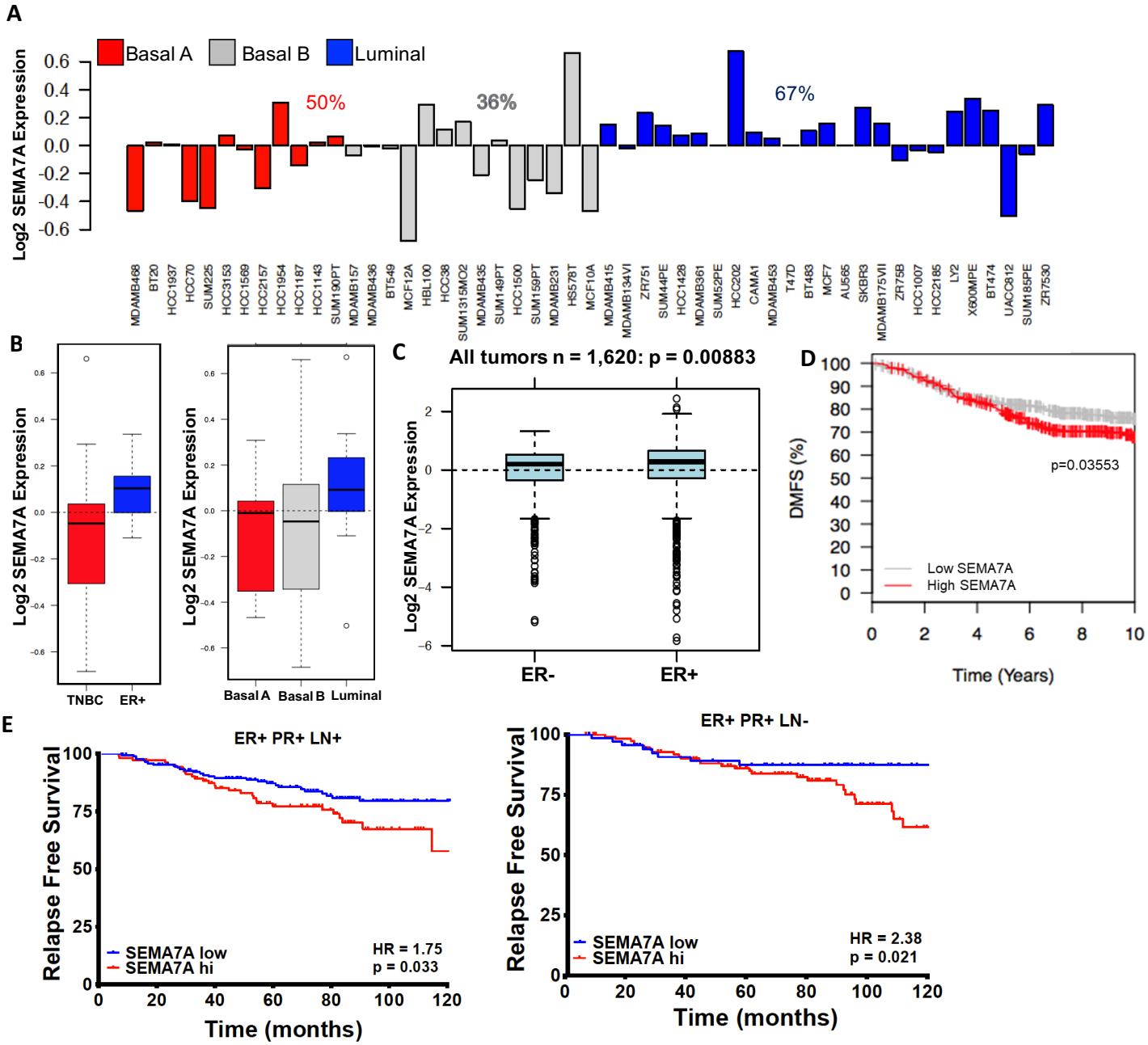

A
Basal A
Basal B
Luminal
Log2 SEMA7A Expression
50%
36%
67%
B
TNBC
ER+
Basal A
Basal B
Luminal
C
All tumors n = 1,620: p = 0.00883
ER-
ER+
D
DMFS (%)
p=0.03553
Low SEMA7A
High SEMA7A
Time (Years)
E
ER+ PR+ LN+
Relapse Free Survival
SEMA7A low
SEMA7A hi
HR = 1.75
p = 0.033
Time (months)
ER+ PR+ LN-
HR = 2.38
p = 0.021

Figure 2

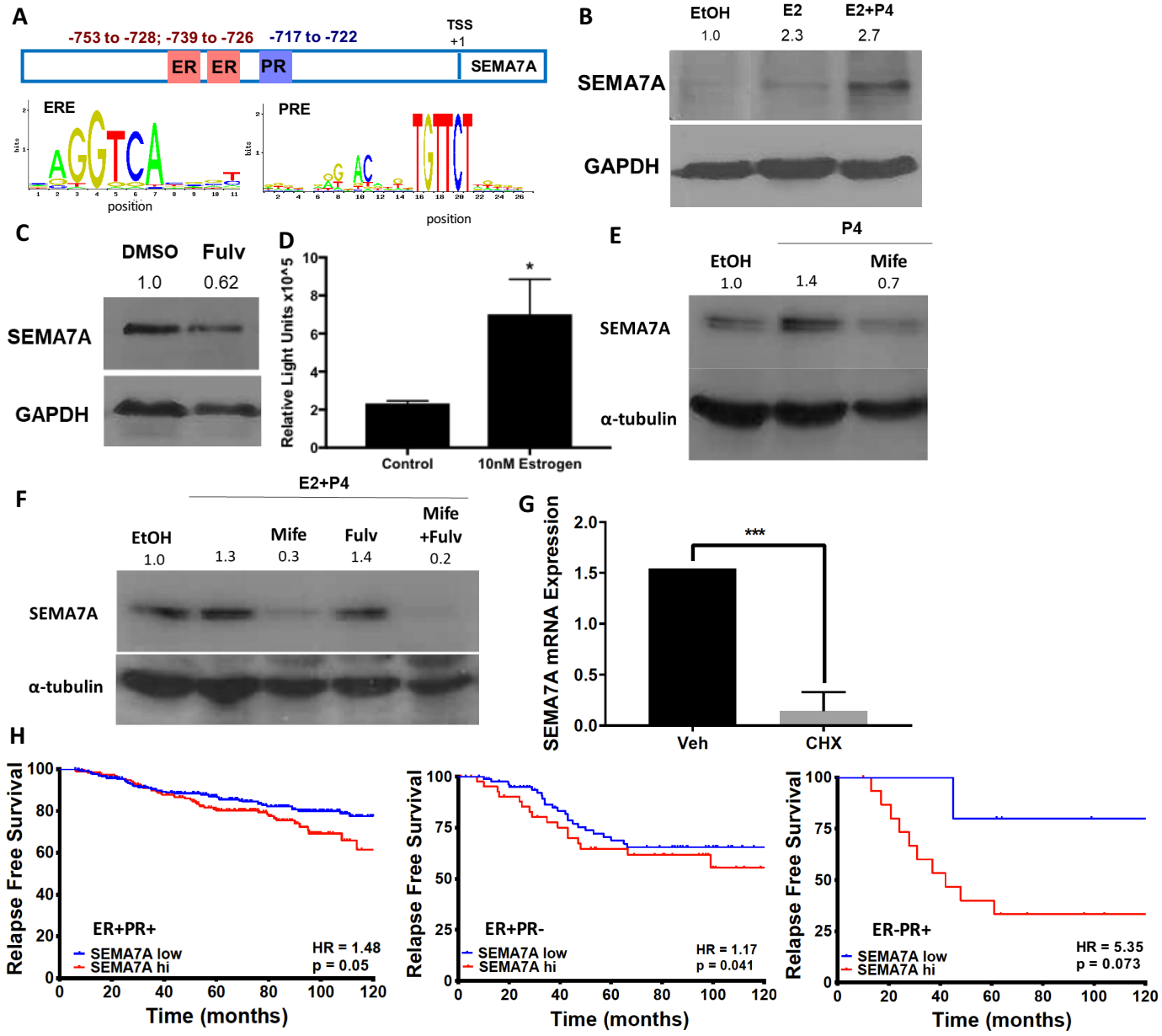

A
-753 to -728; -739 to -726
-717 to -722
TSS
+1
ER
ER
PR
SEMA7A
ERE
PRE
position
position
B
EtOH
E2
E2+P4
1.0
2.3
2.7
SEMA7A
GAPDH
C
DMSO
Fulv
1.0
0.62
SEMA7A
GAPDH
D
Relative Light Units x10^5
Control
10nM Estrogen
E
P4
EtOH
Mife
1.0
1.4
0.7
SEMA7A
α-tubulin
F
E2+P4
EtOH
Mife
Fulv
Mife +Fulv
1.0
1.3
0.3
1.4
0.2
SEMA7A
α-tubulin
G
SEMA7A mRNA Expression
Veh
CHX
H
Relapse Free Survival
Time (months)
ER+PR+
SEMA7A low
SEMA7A hi
HR = 1.48
p = 0.05
ER+PR-
HR = 1.17
p = 0.041
ER-PR+
HR = 5.35
p = 0.073

Figure 3

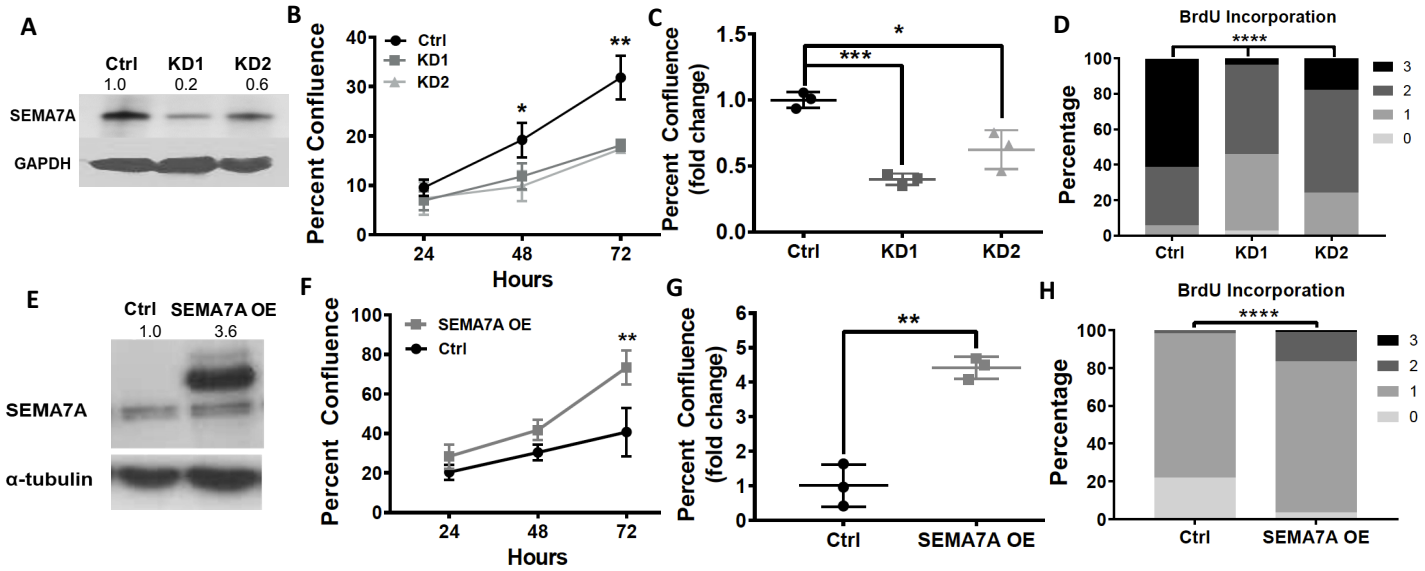

A
Ctrl
KD1
KD2
1.0
0.2
0.6
SEMA7A
GAPDH
B
Ctrl
KD1
KD2
Percent Confluence
Hours
24
48
72
C
Percent Confluence (fold change)
Ctrl
KD1
KD2
D
BrdU Incorporation
Percentage
Ctrl
KD1
KD2
E
Ctrl
SEMA7A OE
1.0
3.6
SEMA7A
α-tubulin
F
SEMA7A OE
Ctrl
Percent Confluence
Hours
G
Percent Confluence (fold change)
Ctrl
SEMA7A OE
H
BrdU Incorporation
Percentage
Ctrl
SEMA7A OE

Figure 4

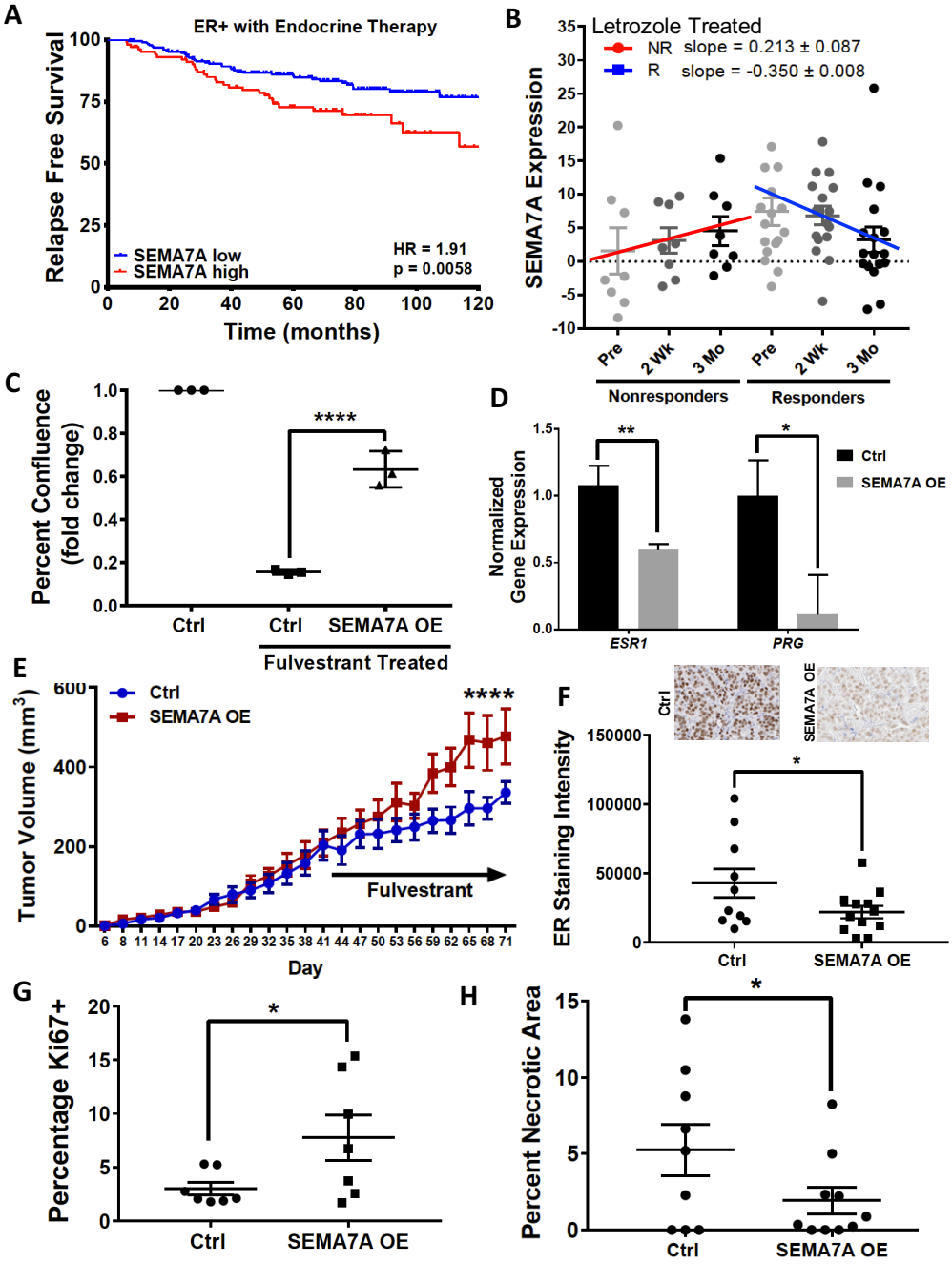

A
ER+ with Endocrine Therapy
Relapse Free Survival
SEMA7A low
SEMA7A high
HR = 1.91
p = 0.0058
Time (months)
B
Letrozole Treated
NR slope = 0.213 ± 0.087
R slope = -0.350 ± 0.008
SEMA7A Expression
Pre
2 Wk
3 Mo
Nonresponders
Responders
C
Percent Confluence (fold change)
****
Ctrl
SEMA7A OE
Fulvestrant Treated
D
Normalized Gene Expression
**
*
Ctrl
SEMA7A OE
ESR1
PRG
E
Tumor Volume (mm3)
Ctrl
SEMA7A OE
****
Fulvestrant
Day
F
Ctrl
SEMA7A OE
ER Staining Intensity
*
G
Percentage Ki67+
*
H
Percent Necrotic Area
*

Figure 5

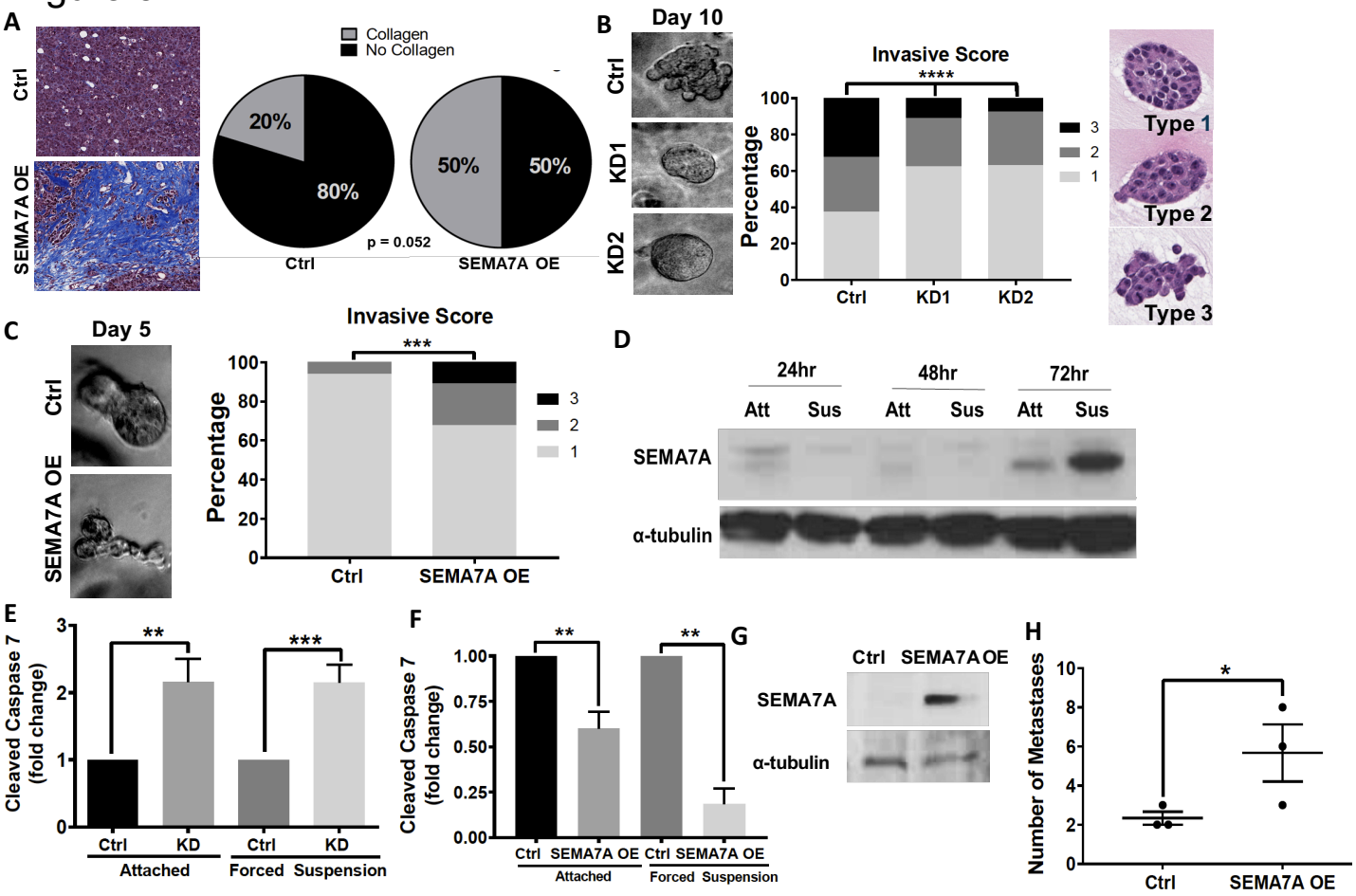
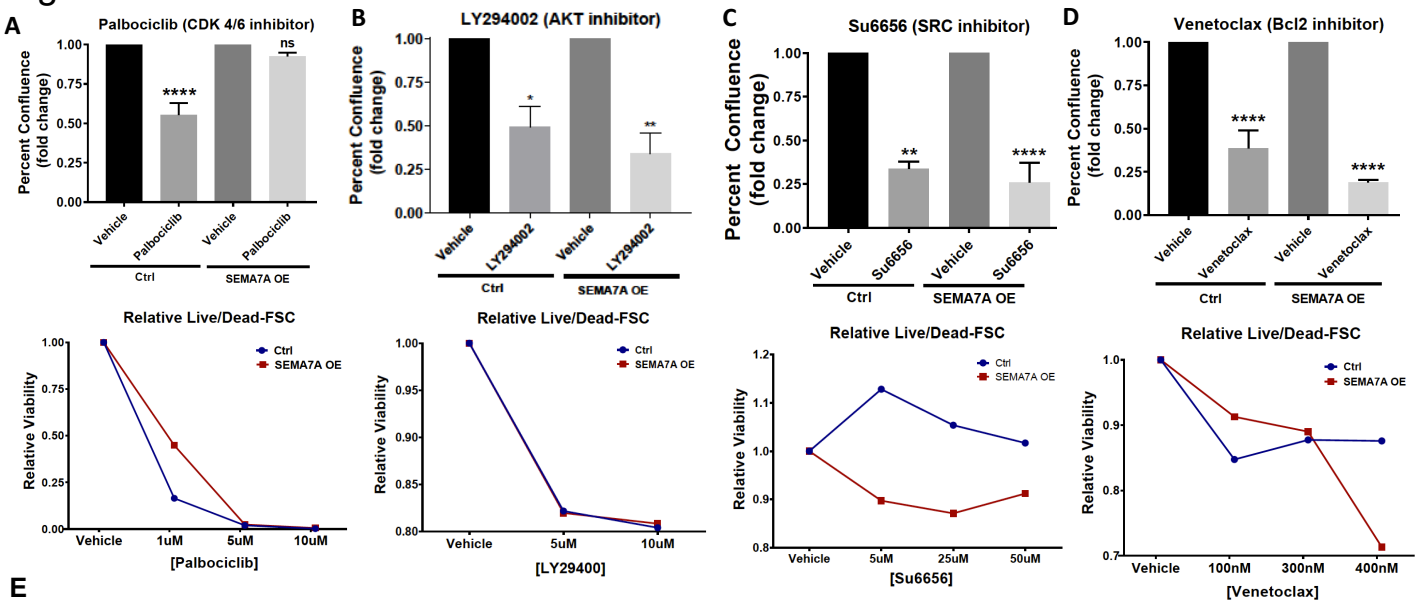

A
Ctrl
SEMA7A OE
Collagen
No Collagen
20%
80%
50%
50%
p = 0.052
Ctrl
SEMA7A OE
B
Day 10
Ctrl
KD1
KD2
Invasive Score
****
Percentage
3
2
1
Type 1
Type 2
Type 3
C
Day 5
Invasive Score
***
D
24hr
48hr
72hr
Att
Sus
SEMA7A
α-tubulin
E
Cleaved Caspase 7 (fold change)
**
***
Attached
Forced Suspension
F
G
H
Number of Metastases
*

Figure 6


A
Palbociclib (CDK 4/6 inhibitor)
Percent Confluence (fold change)
1.00
0.75
0.50
0.25
0.00
****
ns
Vehicle
Palbociclib
Vehicle
Palbociclib
Ctrl
SEMA7A OE
B
LY294002 (AKT inhibitor)
Percent Confluence (fold change)
*
**
Vehicle
LY294002
Vehicle
LY294002
Ctrl
SEMA7A OE
C
Su6656 (SRC inhibitor)
Percent Confluence (fold change)
**
****
Vehicle
Su6656
Vehicle
Su6656
Ctrl
SEMA7A OE
D
Venetoclax (Bcl2 inhibitor)
Percent Confluence (fold change)
****
****
Vehicle
Venetoclax
Vehicle
Venetoclax
Ctrl
SEMA7A OE
Relative Live/Dead-FSC
Relative Viability
Ctrl
SEMA7A OE
Vehicle
1uM
5uM
10uM
[Palbociclib]
Relative Live/Dead-FSC
Relative Viability
Ctrl
SEMA7A OE
Vehicle
5uM
10uM
[LY29400]
Relative Live/Dead-FSC
Relative Viability
Ctrl
SEMA7A OE
Vehicle
5uM
25uM
50uM
[Su6656]
Relative Live/Dead-FSC
Relative Viability
Ctrl
SEMA7A OE
Vehicle
100nM
300nM
400nM
[Venetoclax]

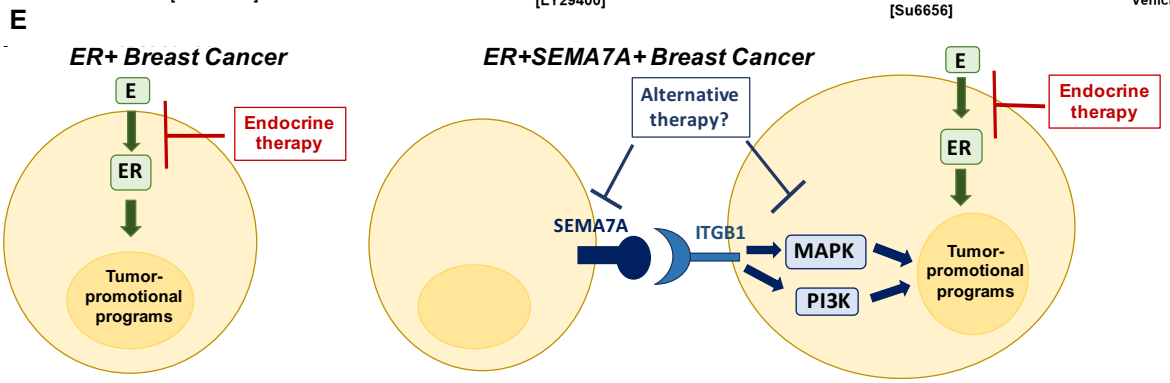

E
ER+ Breast Cancer
E
ER
Endocrine therapy
Tumor-promotional programs
ER+SEMA7A+ Breast Cancer
Alternative therapy?
SEMA7A
ITGB1
MAPK
PI3K
E
ER
Endocrine therapy
Tumor-promotional programs

Table 1

A

Dataset	n	Biomarker Stratification	Highest In	p-value	Fold Change	OE Gene/CN Rank
TCGA2 (copy number)	1602	ER- vs ER+	ER+	0.045	1.027	Top 38%
Ivshina	289	ER- vs ER+	ER+	0.017	1.062	Top 16%
Wang	286	ER- vs ER+	ER+	0.0001	1.156	Top 16%
Lu	129	ER- vs ER+	ER+	0.032	1.275	Top 28%
Nikolsky (copy numbner)	191	ER- vs ER+	ER+	0.012	1.436	Top 3%
Zhang (copy number)	313	ER- vs ER+	ER+	0.04	1.024	Top 31%
TCGA2 (copy number)	1602	PR- vs PR+	PR+	0.007	1.036	Top 27%

B

Dataset	n	Groups	Highest In	p-value	Correlation or Fold Change	OE Gene/CN Rank
TCGA (IDC)	593	Stage I-IV	IV	0.008	0.013	Top 6%
Boersma	95	Stage I-III	III	0.029	0.297	Top 4%
Bittner	336	Stage I-III	III	0.022	0.4	Top 3%
Curtis (IDC)	2136	Stage I-III	III	2.45E-05	0.104	Top 22%
vantVeer	117	Grade 1-3	3	0.019	0.193	Top 12%
Bittner (ductal)	336	Grade 1-3	3	0.047	0.114	Top 31%
Zhao	64	Grade 1-3	3	0.019	0.341	Top 16%
Bittner (mucinous)	336	Grade 1-2	2	0.044	1.953 (fold)	Top 13%
Ma 3	60	Grade 1-3	3	0.003	0.379	Top 3%
Curtis 2 (copy number)	1992	Grade 1-3	3	0.024	0.315	Top 2%
Ma 2	40	Grade 2-3	3	0.011	1.23 (fold)	Top 3%
Bonnefoi	160	Grade 2-3	3	0.012	1.07 (fold)	Top 3%
Ginestier	55	Grade 2-3	3	0.009	1.227 (fold)	Top 8%
Loi 3	77	Elston grade 1-3	3	3.71E-04	0.431	Top 3%
Lu	129	Bloom-Richardson grade 1-3	3	0.027	0.449	Top 7%
Sorlie	167	Node negative vs node positive	N1+	0.041	1.322 (fold)	Top 4%

C

Dataset	n	Drug	Target	Sens/Resist	p-value	SEMA7A Fold Change
Wooster	318	GSK1059615	PI3K	Sensitive	0.008	1.262
Garnett	732	Pictilisib (GDC-0941)	PI3K	Sensitive	0.036	1.084
Barretina 2	947	CHIR-265	RAF	Sensitive	0.007	1.038
Barretina 2	947	PD0325901	MEK	Sensitive	0.007	1.047
Bild 5	19	SU6656	Src	Sensitive	0.018	1.675