TITLE: Semaphorin 7a dependent programs of cell survival, matrix remodeling, and EMT drive breast (postpartum?) tumor progression

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ABSTRACT: Breast cancer (BC) remains the second leading cause of cancer related deaths in women in the US -- mainly due to metastatic disease. Thus, understanding how BC progresses is of the utmost importance to developing new strategies to block metastasis. Young women diagnosed with BC generally have poor prognosis due to increased rates of metastasis. Additionally, women who are within 5 years of most recent child-birth at diagnosis are ~3 times more likely to develop metastasis than age and stage matched nulliparous women. We define these cases as postpartum breast cancers (PPBC) and propose that the unique biology of the postpartum mammary gland drives tumor progression and metastasis. Semaphorin 7a (SEMA7A) is a unique member of the Semaphorin family as it is the only GPI-anchored member that can be shed into the extracellular environment. Our published results show that SEMA7A expression is associated with decreased overall survival in BC patient cohorts and revealed roles for SEMA7A in breast tumor cell growth, motility, invasion, and tumor associated-lymphangiogenesis all of which are also increased in pre-clinical models of PPBC. However, whether primary SEMA7A driver PPBC progression remains largely unexplored. We utilized a pre-clinical of PPBC with breast cancer cells where we silenced or overexpressed SEMA7A. Our results show that silencing of SEMA7A decreases tumor growth in postpartum hosts while overexpression increases growth in nulliparous hosts to rates closer to those observed in postpartum hosts. Further, we show that SEMA7A effects multiple pro-metastatic tumor attributes such as cell survival, tumor associated COX-2 expression, fibronectin deposition, and fibroblast-mediated collagen deposition in the tumor microenvironment regardless of whether the host is nulliparous or postpartum. Finally, we show that co-expression of SEMA7A/Collagen/COX2/FN mRNA predicts for increased metastasis in breast and ovarian cancer cohorts. These studies suggest SEMA7A as a key mediator of general BC progression and that targeting SEMA7A alone or in combination may open avenues for novel therapeutic strategies to prevent BC progression to metastasis.

INTRODUCTION: Postpartum breast cancers (PPBC), or breast cancers diagnosed within 5-10 years of last childbirth, are nearly three times as likely to become metastatic and result in death from breast cancer[1-3]. Specifically, PPBC patients diagnosed within five years of last competed pregnancy exhibit 70% distant metastasis free five-year survival (DMFS) rates[1] that are further decreased to 50% after ten years[2]. Using this definition, PPBC may account over half of breast cancers diagnosed in women aged <45 in two independent cohorts. Using a pre-clinical model of PPBC we have shown that non-invasive breast tumor cells, MCF10DCIS, become invasive and metastatic if they are implanted into mouse mammary tissues during the normal physiological process of postpartum mammary gland involution[4]. The MCF10DCIS model closely resembles ductal carcinoma in situ (DCIS) and progresses to invasive ductal carcinoma (IDC) similar to what is seen in some breast cancer patients[5]. This model allows for monitoring early events in the metastatic cascade such as the transition from in situ to invasive disease and xenograft studies have revealed that postpartum/post-lactational changes to the mammary tissue microenvironment can promote invasion and metastasis in this model[4,6,7].

Postpartum mammary involution is a tissue-remodeling event that returns the gland to the pre-pregnant state after childbirth and/or lactation. We and others have shown that programs associated with mammary gland involution are similar to wound healing and tumor-promotional microenvironments[1,4,6,8-16], which led us to investigate whether the tissue microenvironment created during postpartum involution contributes to breast cancer tumorigenesis and progression. Previously, we published that MCF10DCIS xenografts in recently-weaned or postpartum mice exhibit accelerated growth and progress from *in situ* to invasive disease at three versus four weeks when compared to those injected into nulliparous hosts[4]. This progression is driven, in part, by collagen deposition and collagen mediated expression of COX-2, which promotes tumor cell invasion. Recently, we have shown that Semaphorin 7a (SEMA7A) expression is increased in mouse and human mammary tissues after pregnancy, in the mammary epithelium during the tissue remodeling phase of involution, and in tumor cells after implantation during postpartum involution[9]. Additionally, MCF10DCIS cells stably transfected with shRNAs designed to silence SEMA7A in human derived breast cancer cell lines exhibit decreased attachment and invasion in vitro and decreased in vivo growth and invasion in nulliparous hosts[17]. SEMA7A was first recognized for its expression on lymphocytes and was designated CD108w[18]. Subsequently, CD108w was termed SEMA7A due to its structural similarities with members of the Semaphorin family of proteins. Semaphorins are proteins characterized for their roles in axon guidance and immunemodulaion, however, roles for semaphorins have also been proposed in cancer[19,20]. Structurally, the SEMA7A protein has both Sema and PSI domains, which are characteristic of all semaphorins; SEMA7A also has an Ig-like domain and is linked to the membrane via a glyocosylphospahtidl-inositol (GPI) anchor[21]. The GPI anchor of SEMA7A can be cleaved to result in shedding of SEMA7A into the extracellular environment, which promotes β1-integrin dependent mechanisms of inflammation and fibrosis[22-27]. In fibrosis, TGFβ induces SEAM7A and β1 integrin expression, which activates AKT signaling to result in upregulation of extracellular matrix (ECM) molecules such as collagen and fibronectin to promote formation of fibrotic lesions[28]. Furthermore, in cancer, SEMA7A-β1-integrin binding promotes tumor growth, tumor cell epithelial to mesenchymal transition (EMT), migration, invasion, metastasis, and neo-vasculogenesis[29-31] . Finally, SEMA7A gene expression is increased in breast cancer datasets where expression associates with decreased overall survival[17].

Here, we show that SEMA7A protein is expressed in both in situ and invasive breast cancer patient samples, is necessary for postpartum tumor progression in our pre-clinical model of PPBC, and sufficient to drive tumor progression in non-postpartum hosts. We also demonstrate a connection between collagen, COX-2, and SEMA7A. Specifically, we show that SEMA7A is sufficient to drive collagen deposition in the tumor microenvironment (TME) via upregulation of collagen I mRNA expression by fibroblasts, which results in tumor cell expression of COX-2 and invasion. Furthermore, we propose a pro-survival role for SEMA7A through alterations in the ECM that is mediated through the activation/phosphorylation of AKT. Finally, we identify SEMA7A dependent expression of another extracellular matrix protein, fibronectin (FN), which is also a characteristic of cells that have undergone EMT. To establish whether this mechanism is at play in aggressive breast cancers, we demonstrate that a gene signature of SEMA7A, PTGS2 (encodes for COX-2), and FN1 results in significantly decreased distant metastasis free survival in breast cancer patients compared to FN alone, which is a known mediator of poor prognosis. Taken together, our results suggest that SEMA7A plays a

role in progression of PPBC through activation of pro-invasion and pro-survival programs in breast cancer, which merits further studies to develop a novel therapeutic for breast cancer patients.

RESULTS

SEMA7A protein is highest in DCIS from PPBC patients and promotes tumor growth via activation of cell survival mechanisms

To investigate expression levels of SEMA7A protein in normal breast tissue and breast cancer from patients we performed immunohistochemistry (IHC) on a tissue array consisting of surgical specimens from normal adjacent breast and ductal carcinoma *in situ* (DCIS) lesions from the same patient. Samples from kidney and placenta were included in the array as negative and positive controls, respectively (SFigure 1). We observed that expression levels of SEMA7A, as measured by percent positive pixels within the areas defined as normal breast or DCIS, were significantly increased in the DCIS compared to normal breast tissue (Figure 1A). Then, we examined SEMA7A expression in DCIS from our young women's breast cancer (YWBC) cohort and observed overall higher percent positivity for SEMA7A in DCIS when compared to our analysis of the tissue array, ~40% versus ~12% respectively (Figure 1A versus 1B). To determine whether expression levels in postpartum patients are driving the increase observed in this young cohort we separated the DCIS by never pregnant (nulliparous) and within 5 years of most recent childbirth (postpartum -- PP) and observed 40% positivity for SEMA7A in the postpartum group versus 30% in nulliparous (Figure 1B). These results suggest that SEMA7A is highest in DCIS in postpartum women, but higher in young women in general, where it may be involved in DCIS progression.

To examine whether SEMA7A drives tumor growth in our established pre-clinical model of postpartum DCIS progression, we orthotopically injected MCF10DCIS cell lines that stably express SEMA7A targeted shRNA or nontargeting control[17] at involution day 1 or into nulliparous controls. We observed that silencing of SEMA7A is sufficient to decrease tumor growth in postpartum hosts (Figure 2A). Due to the absence of selective pressure in vivo, some tumor cells regained expression of SEMA7A, however, at study end expression of SEMA7A in the knockdown group remained significantly lower than the control group (Figure S2A). To determine whether SEMA7A expression alone is sufficient to drive DCIS progression in nulliparous hosts, we injected MCF10DCIS cells engineered to express ~4-fold higher SEMA7A protein (SEMA7A OE), along with vector only controls, and observed that SEMA7A overexpressing tumors exhibited increased/accelerated growth (Figure 2B). It is widely recognized that tumor growth is driven by activation of pro-proliferative and/or pro-survival pathways and we have previously shown that postpartum tumors and SEMA7A expressing tumors are more proliferative in independent studies [4,17]. To examine if SEMA7A promotes tumor growth via proliferation in our postpartum model, we stained and analyzed MCF10DCIS control and knockdown tumors for Ki67 by quantitative IHC. Surprisingly, we did not see any significant changes in Ki67 with the knockdown of SEMA7A nor with the overexpression of SEMA7A (Figure S2B). We then examined cleaved caspase 3, as a marker of apoptosis, we observed a significant increase with SEMA7A knockdown in both postpartum and nulliparous tumors suggesting that SEMA7A promotes survival (Figure 2C). We then examined cleaved caspase 3/7 in our knockdown and overexpressing cells in vitro using a luminescence assay. Silencing of SEMA7A significantly increased cleaved caspase 3/7, whereas overexpression of SEMA7A significantly reduced cleaved caspase 3/7 in the MCF10DCIS cell lines as well as in an addition triple negative breast cancer cell line, MDA231 (Figure 2D&E). We also measured cell growth and death in real time to show that silencing of SEMA7A does not decrease growth (Figure S2C) but does increase cleaved caspase-3/7 (Figure 2F). One known mechanism of cell survival co-opted by tumors cells is activation of pro-survival kinase Akt. Thus, we measured levels of activated Akt via immunoblot for p-S473 and observed decreased levels with silencing of SEMA7A and

increased levels with overexpression of SEMA7A (Figure 2G). Thus, we suggest that SEMA7A promotes survival of breast cancer cells via activation of pro-survival signaling mediated by AKT.

SEMA7A drives collagen mediated expression of COX-2 to promote tumor cell invasion

To examine whether SEMA7A drives DCIS progression to IDC in our postpartum model, tumors were scored for invasion. When invasion was scored, 87% of the tumors in the shRNA control group progressed to full IDC compared to only 33% of the shSEMA7A (KD). Further, only 12.5% of control tumors maintained evidence of DCIS, whereas 56% of tumors in the shSEMA7A group remained DCIS-like. Finally, while none of the control tumors were maintained in a non-invasive state, 11% of tumors in the shSEMA7A group remained DCIS with evidence only of microinvasion (Figure 3A). Next, we tested whether SEMA7A OE tumors harvested three weeks after injection, when tumors in nulliparous hosts normally remain DCIS (SFigure 3A), would exhibit accelerated invasion. We observed that greater than 50% of the tumors from the SEMA7A OE cells exhibited evidence of microinvasion and/or IDC (Figure 3B) while the control group remained DCIS as expected. These results suggest that SEMA7A promotes DCIS invasion in postpartum hosts and is sufficient to promote invasion in non-postpartum hosts.

Collagen mediated upregulation of COX-2 is a dominant feature of tumor cell invasion in our postpartum model. To test whether postpartum tumors with decreased invasion and SEMA7A also exhibit decreased COX-2, we examined COX-2 expression by quantitative IHC in our shSEMA7A tumors. We observed significantly decreased percent positivity for COX-2 in our knockdown tumors in postpartum hosts, along with consistently upregulated COX-2 in our SEMA7A OE tumors in nulliparous hosts (Figure 3C&D). To determine if this increase in COX-2 was due to collagen or SEMA7A expression, we first quantitated fibrillar collagen (blue) in postpartum tumors and observed that silencing of SEMA7A decreases the intensity of the collagen stain. Interestingly, overexpression of SEMA7A did not increase the intensity of the stain, but resulted in increased overall collagen content (Figure 3E&F, SFigure 3B&C). To determine the contribution of SEMA7A to invasion collagen-COX-2 dependent invasion where we have shown that collagen mediated upregulation of COX-2 promotes invasion. We found that SEMA7A overexpression alone was insufficient to drive increased invasion (Figure 3G). Similarly, we observed that shSEMA7A and SEMA7A OE cells do not exhibit alterations in COX-2 expression (SFigure 3D). However, with the addition of collagen overexpression of SEMA7A was sufficient to increase the incidence of invasive organoids (Figure 3H). We further confirmed this relationship using the MDA231 model, which is more invasive, to demonstrate that knockdown of SEMA7A was sufficient do decrease the incidence of invasive organoids on collagen (Figure 3I).

Since fibroblasts are the main producers of collagen in the TME, we examined whether SEMA7A dependent fibroblast infiltration occurs in our tumors, but observed no differences (SFigure 4A). Then, we measured SEMA7A in conditioned medias from postpartum and nulliparous tumor cell populations ex vivo to determine whether postpartum tumors differentially signal to fibroblasts via shedding of SEMA7A. Our results show that postpartum tumor cell populations shed more SEMA7A on average than nulliparous (Figure 4A).Then, we treated cultured fibroblasts with conditioned media from our SEMA7A overexpressing tumor cells and controls, to reveal that conditioned media from SEMA7A overexpressing cells induce *COL1A1s* gene expression in fibroblasts (Figure 4B). To show that SEMA7A specifically drives increased *COL1A1* expression we treated fibroblasts with purified SEMA7A to show a similar increase in COL1A1 expression (Figure 4C). These results suggest SEMA7A as an important mediator of tumor cell invasion on collagen.

SEMA7A drives mesenchymal cell phenotypes

To better understand how SEMA7A may also be altering the tumor microenvironment we performed a mass spectrometry analysis on tumor cell conditioned medias from tumors derived from shSEMA7A or control cells. When we specifically examined peptides collected from secreted proteins we observed striking changes in fibronectin (FN), which was significantly decreased with the silencing of SEMA7A (Figure 5A). We then confirmed decreased FN protein expression by quantitative IHC on our shSEMA7A tumors and controls (Figure 5B). As FN is a well-known mesenchymal marker, we also tested if SEMA7A expression promotes mesenchymal phenotypes, such as cellular morphology, by measuring cell aspect ratios. We predicted that mesenchymal-like cells should have ratios >1 since their elongated phenotype renders their length greater than their width, and that epithelial-like cells, which are more cuboidal, would have a ratio of ~1 (see Ref). Our results reveal that silencing of SEMA7A in MDA-231 cells, which are characterized as mesenchymal, decreased the average aspect ratio from 8 to 3 in MDA-231 cells (Figure 5C). Furthermore, SEMA7A overexpression in the MCD10DCIS model was sufficient to increase the average aspect ratio from 1 to 3 (Figure 5D). To extend our observations we also observed additional downregulation of markers associated with mesenchymal and invasive phenotypes in our ex vivo mass spectrometry analysis including members of the S100 family of proteins, matrix metalloproteinase-2 (MMP2), and vimentin (Figure 5E-L). These results suggest that SEMA7A promotes invasion by promoting expression of mesenchymal-associated proteins.

SEMA7A drives cell survival and metastatic potential via FN

Interestingly, FN signals via integrins, which are upstream of pro-survival pathways, such as AKT. Thus, we hypothesized that the increase in cell death observed in shSEMA7A silenced cells was due to their inability to produce FN and activate pro-survival signaling. To determine if FN could specifically rescue the cell death, we plated shSEMA7A cells on FN, with laminin and collagen as other integrin ligand controls and tissue culture plastic as a negative control, and measured cleaved apoptosis by caspase-3/7. Consistent with our previous results shSEMA7A cells exhibited increased activation of apoptosis on plastic. Surprisingly, increased activation of apoptosis also occurred on laminin and collagen, but not on FN, which resulted in levels of cell death that were similar to the control cells with unaltered SEMA7A expression (Figure 6A). These results suggest that exogenous FN can rescue the cell death phenotype observed in the shSEMA7A cells.

Herein we have established a role for SEMA7A in early breast cancer progression. An additional step in the metastatic cascade is detachment from ECM, extravasation, and survival in circulation (anoikis resistance) to seed metastatic sites. To assess a role for SEMA7A in metastatic seeding we performed anokis resistance assays to determine if SEMA7A promotes cell survival in detached conditions. Similar to our results with attached cells, we observe that SEMA7A promotes cell survival in forced suspension in both MCF10DCIS and MDA 231 cells as measured by cleaved caspase-3/7 assay (Figure 6B&C). Interestingly, the less mesenchymal cells (DCIS) were more susceptible to activation of apoptosis compared to the more mesenchymal MDA231 cells suggesting an additional role for mesenchymal programs in SEMA7A induction of anoikis resistance. Further, similar to our experiments in attached conditions FN is able to rescue the decreased cell survival observed in the shSEMA7A cells in forced suspension (Figure 6X). To determine whether SEMA7A is important for the establishment of distant metastasis we performed tail vein injections of MDA 231 cells expressing control or shSEMA7A targeted shRNA. Our results show a possible decrease in metastatic seeding of the lungs as evidenced by number of metastatic lesions per lung (Figure 6D).

SEMA7A, COX-2, FN signature correlates with increased metastasis in breast cancer patients

In the METABRIC and KmPlot datatsets[17] (SFigure 6A), SEMA7A expression decreases overall survival (OS); however, SEMA7A expression alone does not significantly associate with decreased distant metastasis free survival (DMFS) (SFigure 6B). Additionally, although genes encoding for COX-2, SEMA7A and FN, PTGS2, SEMA7A, and FN1 respectively, are all upregulated in breast cancer compared to normal tissue (SFigureX), only FN gene expression is associated with significantly decreased DMFS, HR=1.3 (95% CI: 1.07-1.58, p=0.0032) (SFigure 6B-D). However, when co-expression of PTGS2, SEMA7A and FN1 were examined by Kaplan-Meier analysis for DMFS we observed that high expression of all three resulted in a very significant increase in risk for metastasis HR=1.9 (95% CI: 1.31-2.74, p=0.00052) with 5 year DMFS rates approaching 70%, which is similar to what we have observed in our postpartum patients[1] (Figure 6E). Further, SEMA7A expression significantly correlated with both FN and COX-2 in all breast cancers, suggesting our observations in postpartum breast cancer may be relevant to all breast cancer cases expressing this signature (Figure 6F&G). Finally, we observe a similar relationship with the three gene signature and metastasis in ovarian tumor datasets suggesting that our observations have implications beyond breast cancer and postpartum breast cancer (Figure 6H).

DISCUSSION

Postpartum breast cancer patients diagnosed within five years of most recent childbirth are at high risk for developing metastasis and comprise nearly 30% of all breast cancers diagnosed in women aged <40 in both US and Norwegian cohorts [1]. Recently, results have extended the increased period of risk for developing metastasis in postpartum patients to ten years, which makes the percentage of young breast cancer patients who are at high risk for metastasis after childbirth, nearly 50%. SEMA7A expression, either alone or in combination with expression of other known pro-tumorigenic molecules, is emerging as poor prognostic indicator for multiple tumor types including breast, lung, and melanoma[9,17,29-33]. Here, we identify potential tumor intrinsic and extrinsic roles for SEMA7A in promotion of PPBC growth and invasion, both of which ultimately drive metastasis. Our results suggest that SEMA7A mediated tumor cell survival, rather than proliferation, promotes the increased tumor size observed in postpartum hosts via activation of pro-survival kinase AKT and fibronectin secretion. Further, we show that the collagen/COX-2 mediated feedback loop that establishes invasion in our postpartum model is broken in the absence of SEMA7A and stimulated by SEMA7A overexpression. Specifically, tumor cell expression of SEMA7A increases collagen deposition in vivo and promotes fibroblasts to upregulate COL1A1 mRNA in vitro. In turn, collagen mediates increases in COX-2 expression, which we have shown to drive tumor cell invasion. We also show that SEMA7A supports tumor cell invasion by inducing mesenchymal phenotypes in breast cancer cells. Finally, we reveal a three-gene signature consisting of PTGS2, SEMA7A, and FN1 that not only correlates with poor prognosis in breast cancer patients, but in patients with ovarian as well. We propose a model in which SEMA7A signaling may support multiple aspects of cancer progression (Figure 7).

Our results showing that SEMA7A promotes pro-survival mechanisms in models of triple negative postpartum breast cancer may provide both mechanistic and clinical insight into why postpartum patients, and all patients with high expression of SEMA7A, exhibit poor survival rates. First, SEMA7A mediated activation of pro-survival AKT signaling may not only cause upregulation of pro-survival signals, but could have additional downstream effects that result in tumor progression such as our observation of increased fibronectin, a known mediator of metastasis, expression. Although not directly addressed herein, we hypothesize that SEMA7A turns on AKT signaling via both secretion of FN and direct binding to β1-integrin, which has been shown to activate PI3K, which goes on to phosphorylate and activate AKT (refs). Activated AKT then phosphorylates and activates multiple downstream pathways associated with cancer progression. Of particular note, AKT can activate NFκB(refs), a transcription factor known for its pro-tumorigenic functions including upregulation of both COX-2

and SEMA7A expression[34,35] and we have previously shown that SEMA7A expression is decreased when COX-2 is silenced[17]. Thus, indicating that AKT and NFκB may also function in the SEMA7A collagen/COX-2 mediated feed-forward loop that promotes cell survival and invasion (Figure 7). Interestingly, AKT activity has been shown to directly promote fibronectin expression in multiple cell types[36-38]. Thus, we think our results showing SEMA7A dependent activation of AKT and increased FN expression in our model of triple negative postpartum breast cancer suggest that this pro-tumorigenic mechanism is at play in postpartum patients with triple negative breast cancer. Clinically, this is important as AKT signaling promotes resistance to many chemotherapeutics, such as cisplatin and paclitaxel, which rely on activation of apoptosis for efficacy. Since cisplatin and paclitaxel are front line therapy for patients with triple-negative breast cancer, the poor prognosis observed in our postpartum patients may be attributed to inherent resistance to chemotherapy due to activation of this SEMA7A dependent mechanism. Additionally, we speculate that if SEMA7A contributes to chemotherapeutic resistance then direct targeting of SEMA7A may be useful for chemo-refractory patients. Finally, in tamoxifen resistant breast cancer cell lines AKT activity also results in upregulation of fibronectin, which in turn promotes therapeutic resistance. Thus, patients in our postpartum cohort with estrogen receptor positive breast cancer, who do not appear to respond favorably to anti-endocrine therapy as they show survival rates as low as those with triple-negative breast cancer [39], may develop resistance to anti-endocrine therapy through a SEMA7A dependent mechanism. This SEMA7A dependent mechanism may be more broadly at play in other female hormone-dependent cancers as our signature of high SEMA7A, COX-2 and FN also predicts poor survival in ovarian cancer, which often develops chemotherapeutic resistance, especially to cisplatin, and estrogen resistance (refs). Interestingly, in a model of neuronal development it was shown that estrogen and/or progesterone may be promoting SEMA7A expression, suggesting a complex signaling network may be occurring in hormone dependent breast cancers with elevated levels of SEMA7A to promote treatment resistance. Further, tamoxifen treated mammary glands expressed decreased levels of fibronectin, collagen, and MMP-2[40], suggesting the pathways we have identified during cancer may also play a role in mammary gland development. Future studies are needed to better understand the complex interplay between SEMA7A, hormones, and subsequent downstream signaling contributes to cancer progression and treatment resistance.

We also identify a novel link between SEMA7A and tumor associated FN and suggest that FN may act downstream of SEMA7A to promote mesenchymal phenotypes, tumor cell invasion, and survival. Functionally, in cancer FN engagement of $\alpha_v\beta_1$ integrin activates the transcription factor Slug, which is known to promote transcription of mesenchymal genes and thus the epithelial to mesenchymal transition (EMT). EMT is also emerging as an important determinant of therapeutic resistance as many of the pathways associated with EMT, such as TGFβ, Notch, Hedgehog and Wnt. Additionally, similar to SEMA7A, EMT induced transcription factors such as Slug and and Zeb1 regulate pro-survival pathways, which results in resistance to chemotherapeutic and targeted therapy treatments. Of note, SEMA7A promotes resistance to EGFR-targeted tyrosine kinase inhibitors (TKIs) in lung adenocarcinoma[41] and lung cancer cells with increased expression levels of EMT markers are less sensitive to TKIs[42,43]. Interestingly, SEMA7A has previously been directly linked to EMT in a Ras-dependent model of murine mammary carcinoma in a TGFβ inducible manner where the authors show that TGFβ is unable to induce an EMT without SEMA7A[29]. Better understanding of how SEMA7A contributes to EMT and resistance will help to develop new targeted therapies for refractory patients.

We further demonstrate that SEMA7A is involved in an additional, but not unrelated, signaling axis that may be established via matrix remodeling through fibroblast mediated production of collagen. Our previous results have shown that collagen deposition during postpartum mammary gland involution results in upregulation of COX-2 in tumor cells, which in turn promotes their invasion and metastasis. Further, fibroblasts are uniquely activated during postpartum involution in a COX-2 dependent manner that display pro-tumorigenic and

immunosuppressive activity[13]. Here, we report that SEMA7A mediates collagen deposition by fibroblasts and in the mammary tumor microenvironment providing novel mechanistic insight into signals that regulate collagen deposition during normal postpartum involution and mammary tumorigenesis. Known roles for collagen, specifically fibrillar collagen, are promotion of invasion, metastasis and drug resistance. Additionally, it has long been recognized that increased breast density increases risk for developing breast cancer. Thus, our results also suggest that SEMA7A expression on the mammary epithelium during involution may promote the increased collagen deposition observed, which may play a role for the increased risk for developing breast cancer that can be attributed to recent pregnancy [1,4,7,39,44-46]. Furthermore, in the normal breast, collagen is typically organized into structures that run parallel to the epithelium. However, during breast cancer progression, and during involution, collagen progressively thickens, becomes linearized, and changes to a perpendicular orientation[45,46]. This orientation of the collagen fibers provides directional routes for tumor cells move along, which facilitates local invasion, vessels extravasation, and eventual metastasis. Interestingly, we show that in the absence of collagen SEMA7A overexpression is not sufficient to increase invasion in three dimensional cultures suggesting that while SEMA7A may alter cells to a more mesenchymal phenotype, collagen in the tumor microenvironment is also required for successful invasion. Collagen deposition also increases the stiffness of the surrounding tissue, which activates mechanosignaling pathways, such as FAK and ERK1/2, which are also regulated by SEMA7A signaling and can facilitate cellular invasion[33,47-49]. Furthermore, collagen crosslinking and degrading enzymes are also upregulated during tumor invasion. One family of enzymes that degrade collagen and have known roles in cancer are the matrix metalloproteinases (MMPs). Within this family, MMP-2 and MMP-9 appear to be regulated by SEMA7A as they are both decreased when SEMA7A is silenced in oral squamous cell carcinoma. Taken together, these data suggest that SEMA7A plays multiple roles in modulating collagen and cell invasion during tumor progression.

Importantly, we show for the first time that SEMA7A protein is expressed at increased levels in patient samples of DCIS. We also have identified SEMA7A as a key mediator of PPBC. Currently, there are no targeted therapies or specific treatment options for women diagnosed with PPBC. We propose that targeting SEMA7A could be a potential therapeutic option for these women with our findings that SEMA7A plays a key role in PPBC. Further, we postulate that SEMA7A targeting is feasible as SEMA7A is not normally expressed at high levels in adults. In fact, analysis of publicly available data sets reveal that SEMA7A expression is elevated across all subtypes of breast cancer, suggesting that a SEMA7A targeted therapy could benefit a large number of breast cancer patients. If direct targeting of SEMA7A is not feasible, SEMA7A activates many downstream targets such as FAK, Src, and ERK, which currently have targeted therapies in various stages of development and clinical trials. Although current and past clinical trials in breast cancer for some of these targeted inhibitors have been unsuccessful, perhaps SEMA7A could serve as a biomarker for patients who have hyper-activation of these pathways and thus benefit from targeted therapies. We also have further identified connections between SEMA7A and COX-2, which also has available targeted drugs available. COX-2 is inhibited by both celecoxib and NSAIDs, both of which have been used in the clinical setting. A better understanding of how COX-2 and SEMA7A interact and what downstream pathways they activate to promote breast cancer progression will be needed, but opens a window of opportunity for novel therapeutic combinations.

MATERIALS AND METHODS

Cell Culture and reagents

MCF10DCIS.com cells were obtained from K. Polyak and A. Marusyk (University of Harvard, Cambridge, MA). MDA-231 cells were obtained from P. Schedin (Oregon Heath and Sciences University, Portland OR). HLF-1 cells were gifted from M. Fini (University of Colorado Anschutz Medical Campus, Denver, CO). Cells were

validated by the DNA sequencing core at the University of Colorado Anschutz Medical campus and identified to be a pure population of their respective cell lines. Cells were regularly tested for mycoplasma throughout studies. Cells were sub-cultured as previously described, or according to ATCC standards[4,50]. shRNA silencing was achieved using shRNA SEMA7A targeting plasmids (SABiosciences, Frederik, MD, and Functinal Genomics Facility at University of Colorado Anschutz Medical Campus, Denver, CO) and confirmed via qPCR and Western blot analysis. Overexpression plasmid (SEMA7A-Fc) was a generous gift from R. Medzhitov (Yale University, New Haven, CT). Control plasmid (pcDNA3.1) was obtained from H. Ford University of Colorado Anschutz Medical Campus, Denver, CO). All other overexpression plasmids (p304-V5-Blasticidin and V5-SEMA7A) were obtained from the Functional Genomics Core at the University of Colorado Anschutz Medical Campus and confirmed via qPCR and Western blot analysis. Purified SEMA7A was isolated from MDA-231 cells engineered to overexpress SEMA7A-Fc in collaboration with the Protein Purification/MoAB/Tissue culture core at the University of Colorado Anschutz Medical Campus. 3D cultures were performed as previously described (Lyons NM, Black 2016).

RNA isolation

RNA was isolated using the Quick-RNA kit (Zymo Research Irvine, CA,). Concentration and purity were assessed using absorbance at 280 nm, 260 nm, and 230 nm. cDNA was synthesized from 1 ug RNA using the Bio-Rad (Hercules, CA) iScript cDNA Synthesis Kit in the following conditions: 25ºC for 5 min, 42ºC for 30 min, and 85ºC for 5 min.

qPCR analysis

qPCR of cDNA samples was performed using the Bio-Rad iTaq Universal SYBR Green Supermix with primers for *SEMA7A, COL1A1, COX-2* (Bio-Rad PrimePCR, Hercules, CA) and *FN1* (forward: GCCAGTCCTACAACCAGTATTC , reverse: CTAAGCTGGGTCTGCTAACATC) 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. The fidelity of our primers was assessed after each qPCR experiment using a 95ºC melt curve. mRNA quantification was normalized using the geometric average of two reference genes, *GAPDH* (forward: CAAGAGCACAAGAGGAA GAGAG, reverse: CTACATGGCAACTGTGAGGAG) and *RPS18* (forward: GCGAGTACTCAACACCAACA, reverse: GCTAGGACCTGGCTGTATTT).

Western blot analysis

Western blots were performed by loading equal amounts of protein into each lane. Protein concentration in lysates was determined by Bradford assay. 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. Phosho-AKT blots were probed with phospho-specific and total antibodies (Cell Signaling Technologies, Danvers, MA) and donkey anti-mouse or anti-rabbit (LiCOR, Lincoln, NE, USA). Blots were imaged and quantitated on an Odyssey CLx Imager (LiCOR).

Animal model

MCF10DCIS.com cells with shSEMA7A or SEMA7A overexpressing constructs were injected into the left and right #4 mammary fat pad of female SCID Hairless Outbread mice from Charles River or Nude athymic mice from Charles river. The postpartum model was used as previously described (Lyons NM).

Histologic analysis

Tumors were harvested at 5 weeks (shSEMA7A studies) or 3 weeks (SEMA7A overexpression studies). Mammary glands with intact tumor were prepared for immunohistochemistry as previously described (cite). Hematoxylin and eosin stained sections of tumors were examined by a board-certified anatomic pathologist

(AN) who was blinded to experimental group. Tumors were scored for invasive as follows: 0-lesions which contained only well-devolved DCIS structures with clearly defined basement membranes and no evidence of microinvasion; 1-lesions that contained extensive DCIS with identifiable micro-invasive foci; 2-lesions that contained significant areas of sheet-like invasive tumor growth and mixed with areas of DCIS; 3-lesions that contained entirely invasive tumor with rare to absent DCIS remnants.

Immunohistochemistry and Immunofluorescence

Antibody information is provided in Supplementary Table X. For FN and COX-2 40X images were taken of intact tumor and quantitated using ImageJ software. For SEMA7A, cleaved caspase-3, and trichrome stain quantification of total tumor area (necrotic and stromal areas removed) and percent positive stain or stain intensity was performed using Image Aperio Analysis software (Leica, Buffalo Grove, IL). Areas for quantification were annotated using Aperio analysis tools and percent weak, medium, and strong determined using the color-deconvolution tool. Immunofluorescent images were obtained using 40X objective on OLYMPUS microscope and quantitated using ImageJ.

Kaplan-Meier survival statistical analysis

Km plotter was used to determine distant metastasis free survival. Briefly, in Km plot for breast cancer, SEMA7A, PTGS2 (COX-2), and FN1 were queried using the multigene classifier. Kaplan-Meier plotter is capable of assessing the effect of 54,675 genes on survival using 10,461 cancer samples. This includes 5,143 breast cancer samples with a mean follow-up of 69 months.

Mass spectrometry analysis

Small tumor pieces (~1mm) were placed on gelatin sponges (Novartis Animal Heath, Greensboro, NC, USA) in serum free media. After 48 hours tumor conditioned media was collected and spun down to remove any cellular debris. Media was then run on tandem mass spectrometry and analyzed for total protein content. Further analysis was performed to determine species specific peptides. (Further details to be added by Hansen lab)

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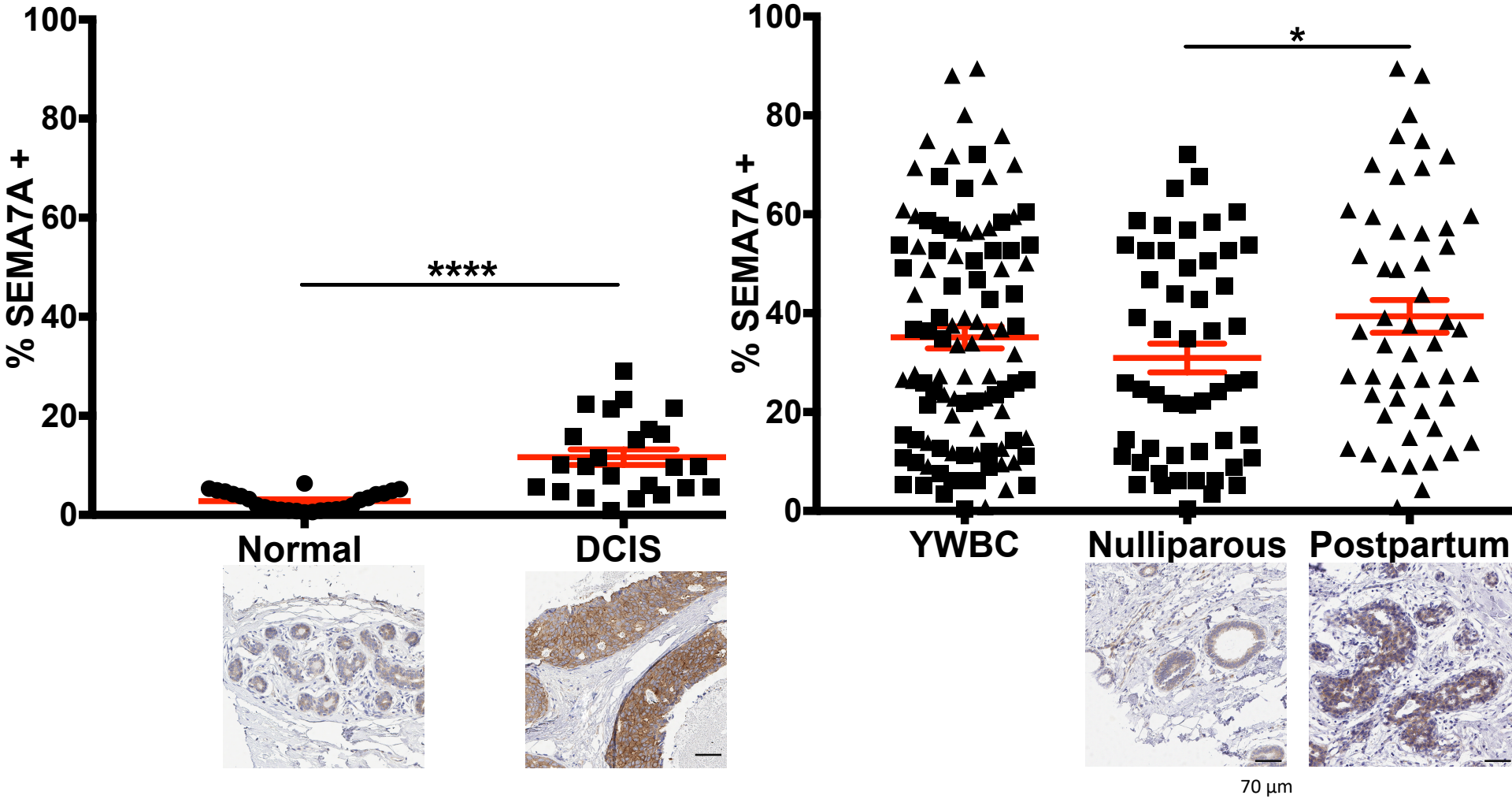
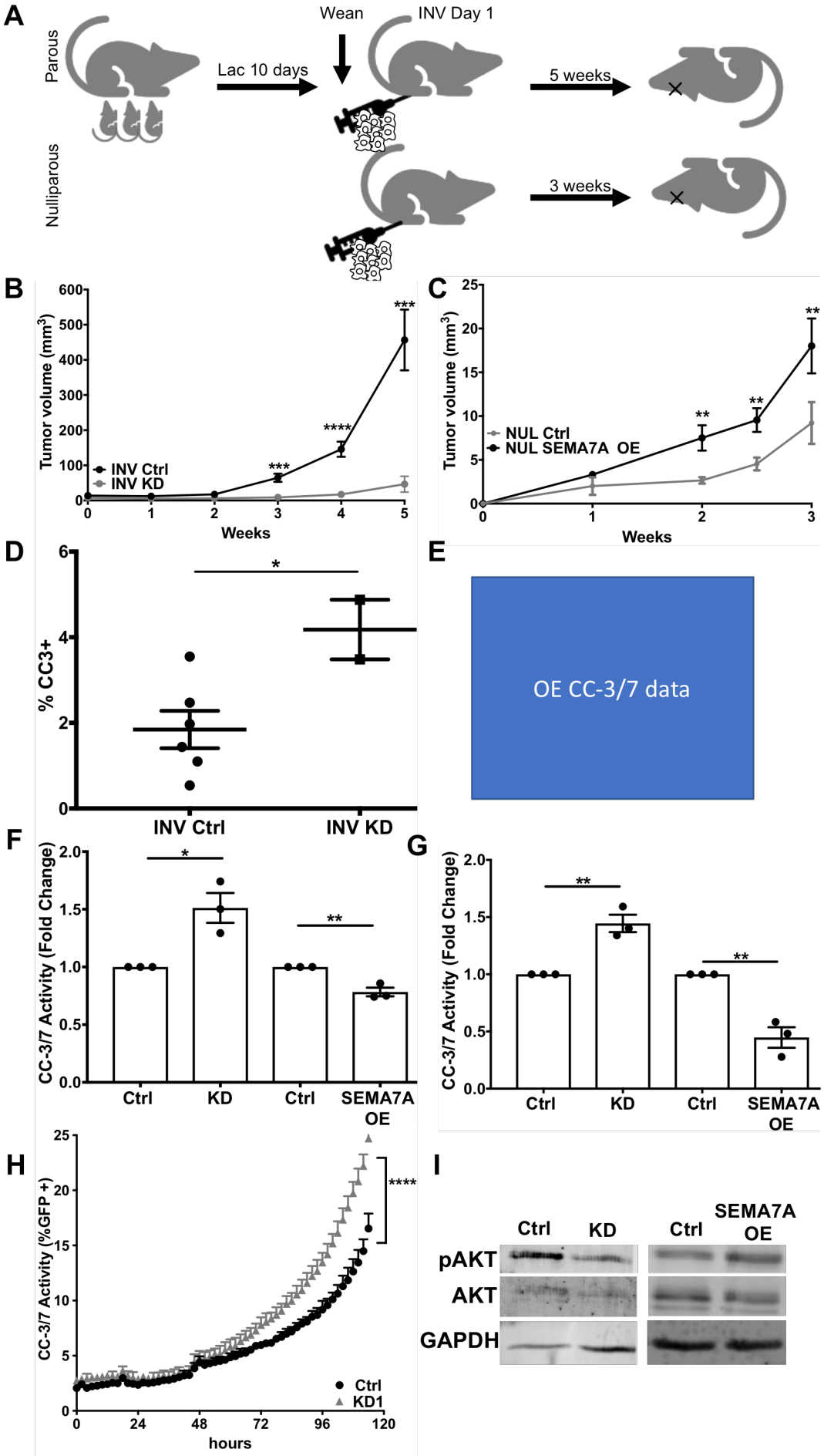

100
80
60
40
20
0
% SEMA7A +
****
Normal
DCIS
100
80
60
40
20
0
% SEMA7A +
*
YWBC
Nulliparous
Postpartum
70 µm


A
Parous
Nulliparous
Lac 10 days
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INV Day 1
5 weeks
3 weeks
B
Tumor volume (mm3)
INV Ctrl
INV KD
Weeks
C
Tumor volume (mm3)
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D
% CC3+
INV Ctrl
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E
OE CC-3/7 data
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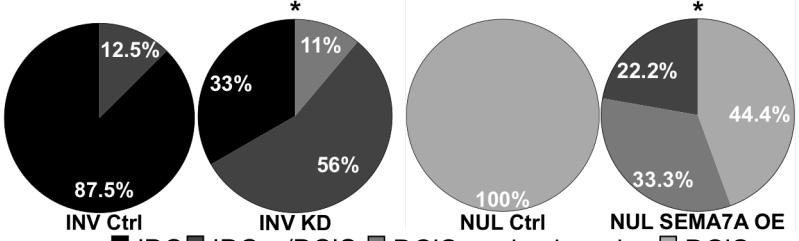
pAKT
AKT
GAPDH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87.5%
INV Ctrl
*
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INV KD
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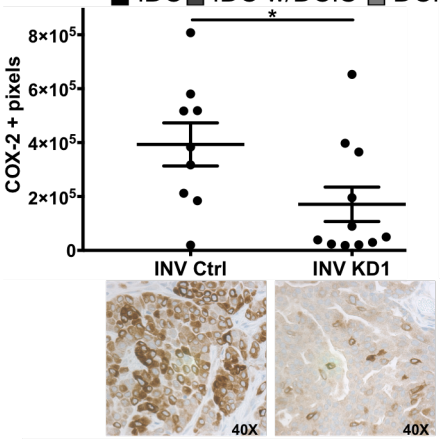
33.3%
NUL SEMA7A OE
IDC
IDC w/DCIS
DCIS + microinvasion
DCIS


COX-2 + pixels
8×10^5
6×10^5
4×10^5
2×10^5
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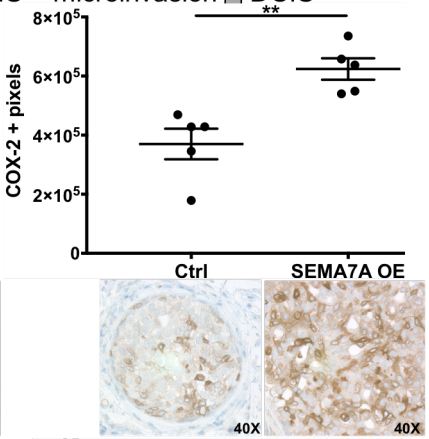
*
INV Ctrl
INV KD1
40X
40X


COX-2 + pixels
8×10^5
6×10^5
4×10^5
2×10^5
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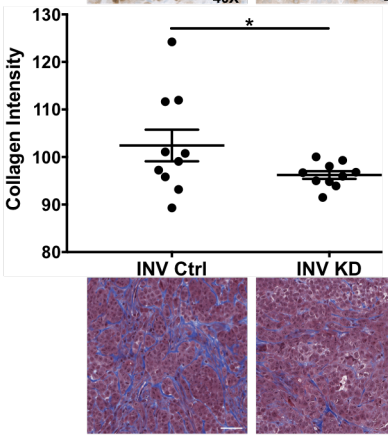
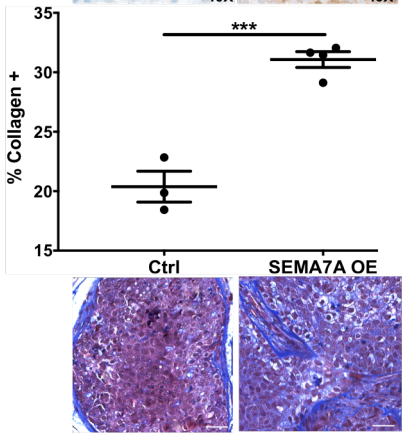
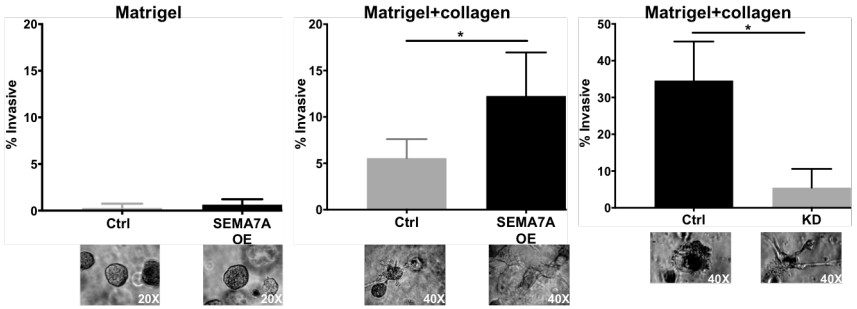
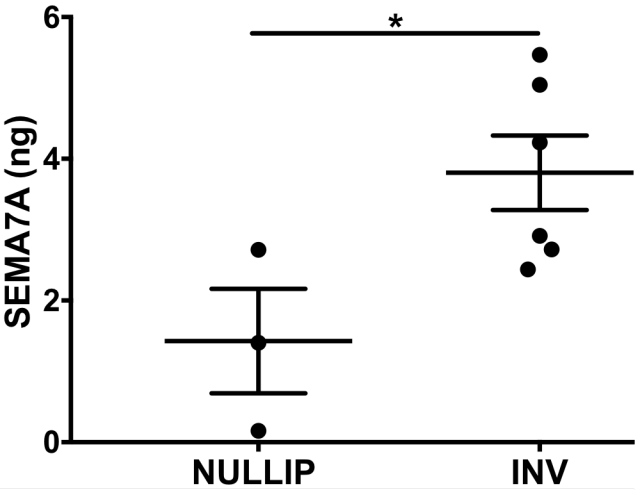
Ctrl
SEMA7A OE
40X
40X


Collagen Intensity
130
120
110
100
90
80
*
INV Ctrl
INV KD


% Collagen +
35
30
25
20
15
***
Ctrl
SEMA7A OE


Matrigel
% Invasive
20
15
10
5
0
Ctrl
SEMA7A OE
20X
20X
Matrigel+collagen
% Invasive
20
15
10
5
0
*
Ctrl
SEMA7A OE
40X
40X
Matrigel+collagen
% Invasive
50
40
30
20
10
0
*
Ctrl
KD
40X
40X


SEMA7A (ng)
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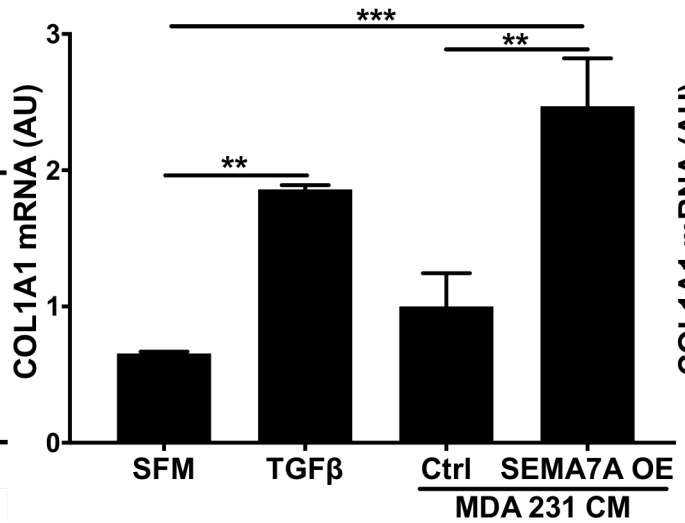
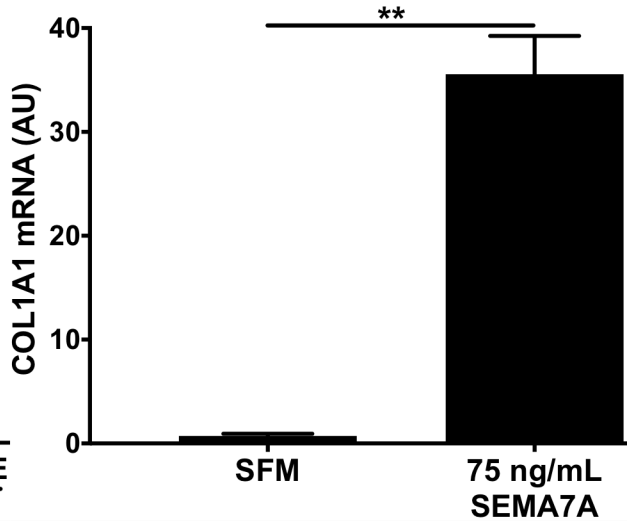
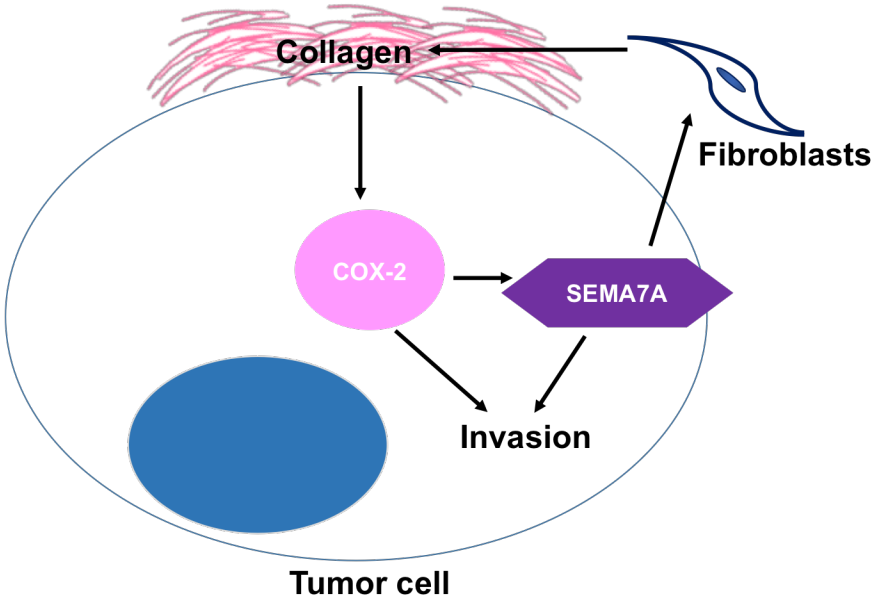
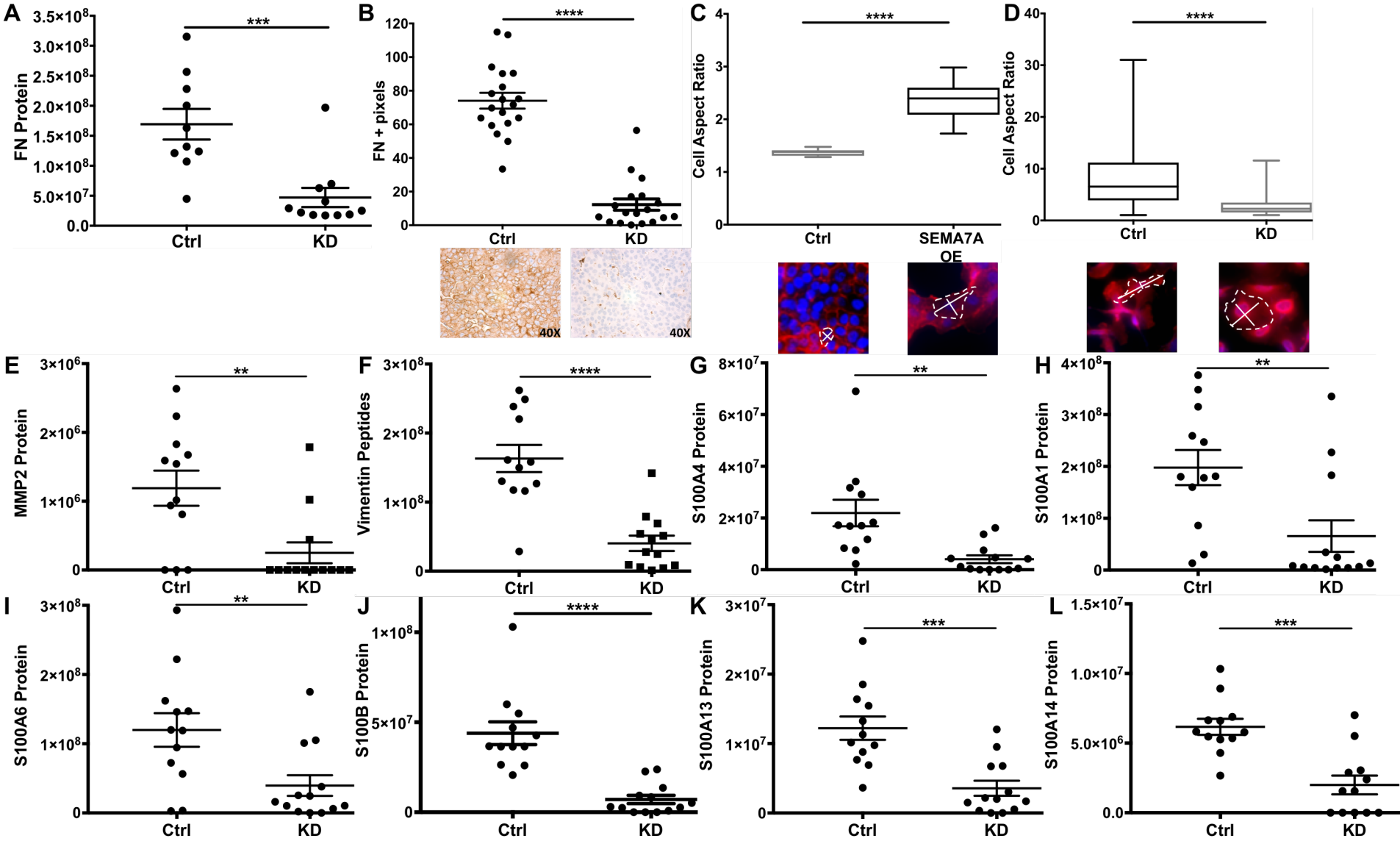
4
2
0
*
NULLIP
INV


COL1A1 mRNA (AU)
3
2
1
0
***
**
**
SFM
TGFβ
Ctrl
SEMA7A OE
MDA 231 CM


COL1A1 mRNA (AU)
40
30
20
10
0
**
SFM
75 ng/mL
SEMA7A


Collagen
Fibroblasts
COX-2
SEMA7A
Invasion
Tumor cell


A
FN Protein
3.5×10^8
3.0×10^8
2.5×10^8
2.0×10^8
1.5×10^8
1.0×10^8
5.0×10^7
0.0
***
Ctrl
KD
B
FN + pixels
120
100
80
60
40
20
0
****
Ctrl
KD
40X
40X
C
Cell Aspect Ratio
4
3
2
1
0
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Ctrl
SEMA7A OE
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Cell Aspect Ratio
40
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MMP2 Protein
3×10^6
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Vimentin Peptides
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S100A4 Protein
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6×10^7
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S100A1 Protein
4×10^8
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S100A6 Protein
3×10^8
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1×10^8
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S100A13 Protein
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2×10^7
1×10^7
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KD
L
S100A14 Protein
1.5×10^7
1.0×10^7
5.0×10^6
0.0
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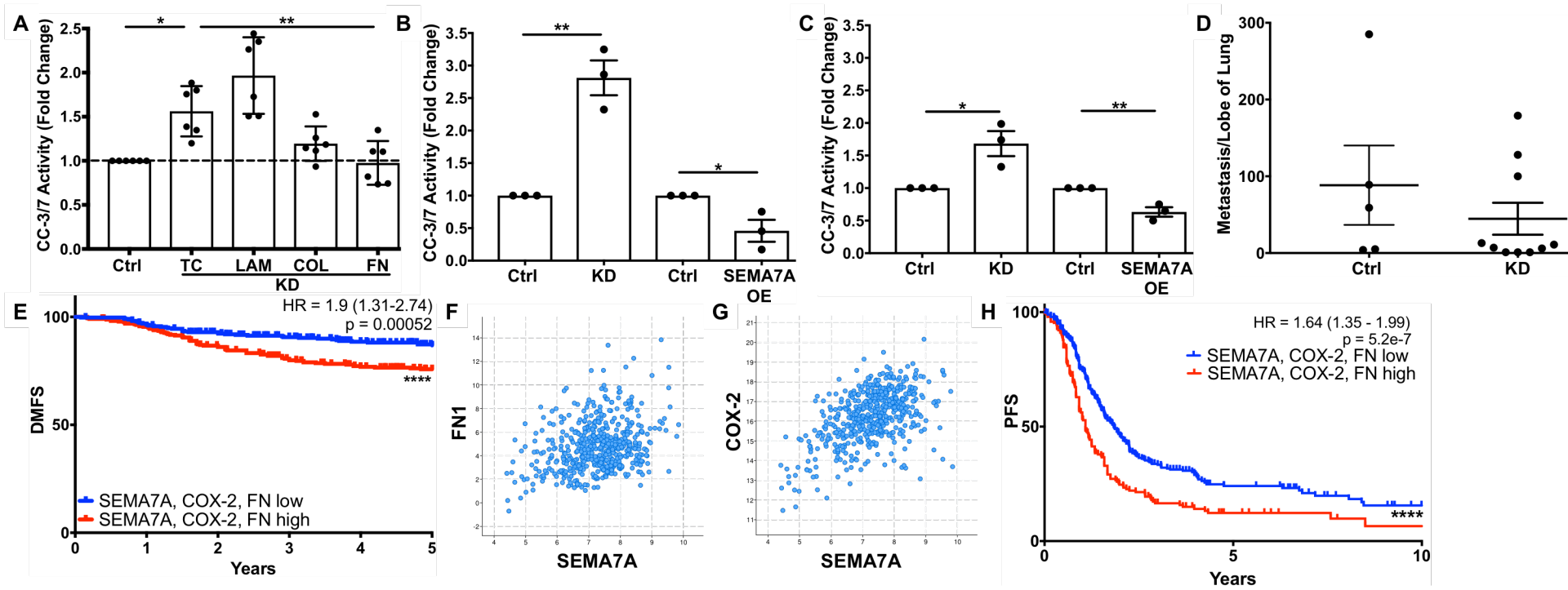
KD


A
CC-3/7 Activity (Fold Change)
Ctrl
TC
LAM
COL
FN
KD
B
CC-3/7 Activity (Fold Change)
Ctrl
KD
Ctrl
SEMA7A OE
C
CC-3/7 Activity (Fold Change)
Ctrl
KD
Ctrl
SEMA7A OE
D
Metastasis/Lobe of Lung
Ctrl
KD
E
HR = 1.9 (1.31-2.74)
p = 0.00052
DMFS
Years
SEMA7A, COX-2, FN low
SEMA7A, COX-2, FN high
F
FN1
SEMA7A
G
COX-2
SEMA7A
H
HR = 1.64 (1.35 - 1.99)
p = 5.2e-7
PFS
Years
SEMA7A, COX-2, FN low
SEMA7A, COX-2, FN high