University of Colorado
Anschutz Medical Campus

A role for SEMA7A in regulating cell invasion and macrophage matrix remodeling during mammary gland morphogenesis

Petra A Dahms[1,3], Taylor Rutherford[1], Traci R Lyons[1,2].

[1]University of Colorado, Anschutz Medical Campus, Department of Medicine, Division of Medical Oncology
[2]University of Colorado Cancer Center Young Women's Breast Cancer Translational Program
[3]University of Colorado, Anschutz Medical Campus, Cancer Biology Graduate Program

Abstract

Approximately 25% of newly diagnosed breast cancer cases are non—invasive or pre-invasive breast cancers known as ductal carcinoma in situ (DCIS). DCIS can progress to invasive ductal carcinoma (IDC), conferring a more dangerous prognosis; however, there is a gap in knowledge regarding what ultimately causes this transition. Understanding epithelial invasion in the mammary gland will provide insight on how DCIS progresses to IDC. Our lab has found that DCIS transforms to IDC under the "wound-healing" pressure of postpartum tissue remodeling–termed postpartum mammary gland involution. This invasive transformation is dependent, in part, on collagen-induced cyclooxygenase (COX2) expression. We identified that a membrane-bound signaling molecule, semaphorin-7a (SEMA7A), promotes both collagen deposition and COX-2 expression and therefore DCIS progression (Tarullo S, et al. *Oncogene* 2020). SEMA7A is also naturally upregulated in mammary epithelial cells during postpartum involution (Rutherford T, et al. *Cell Death & Disease* 2021) and in DCIS relative to normal epithelium (Tarullo 2020). We have identified that inhibition or silencing of SEMA7A can inhibit DCIS invasion (Tarullo 2020). While this demonstrates that SEMA7A expression is sufficient to promote innate capacity for tumor cell invasiveness, we have yet to investigate how SEMA7A interacts with the tumor microenvironment (TME) to promote stromal changes known to advance this transition, such as collagen remodeling or immune cell recruitment. We have demonstrated a potential role for DCIS-associated macrophages in facilitating this transition. We have found that SEMA7A polarizes macrophages in between an M1- and M2-like state and can promote macrophage remodeling of the lymphatic vasculature as well as tumor cell entry into lymphatic vessels (Elder A, et al. *Cancer Res* 2018). Furthermore, we have shown that SEMA7A promotes expression of matrix remodeling enzymes. Our results suggest that SEMA7A mediates DCIS invasion through both intrinsic and extrinsic mechanisms, which could facilitate progression to metastasis. By understanding how SEMA7A regulates DCIS progression we can begin to introduce potential targets for preventative therapies for development of invasive disease–including targeting SEMA7A itself. Since SEMA7A is low in most adult tissues it is expected that such a therapeutic would be less toxic for patients than the current therapies.

Knocking out SEMA7A disrupts normal epithelial invasion during pubertal mammary gland development

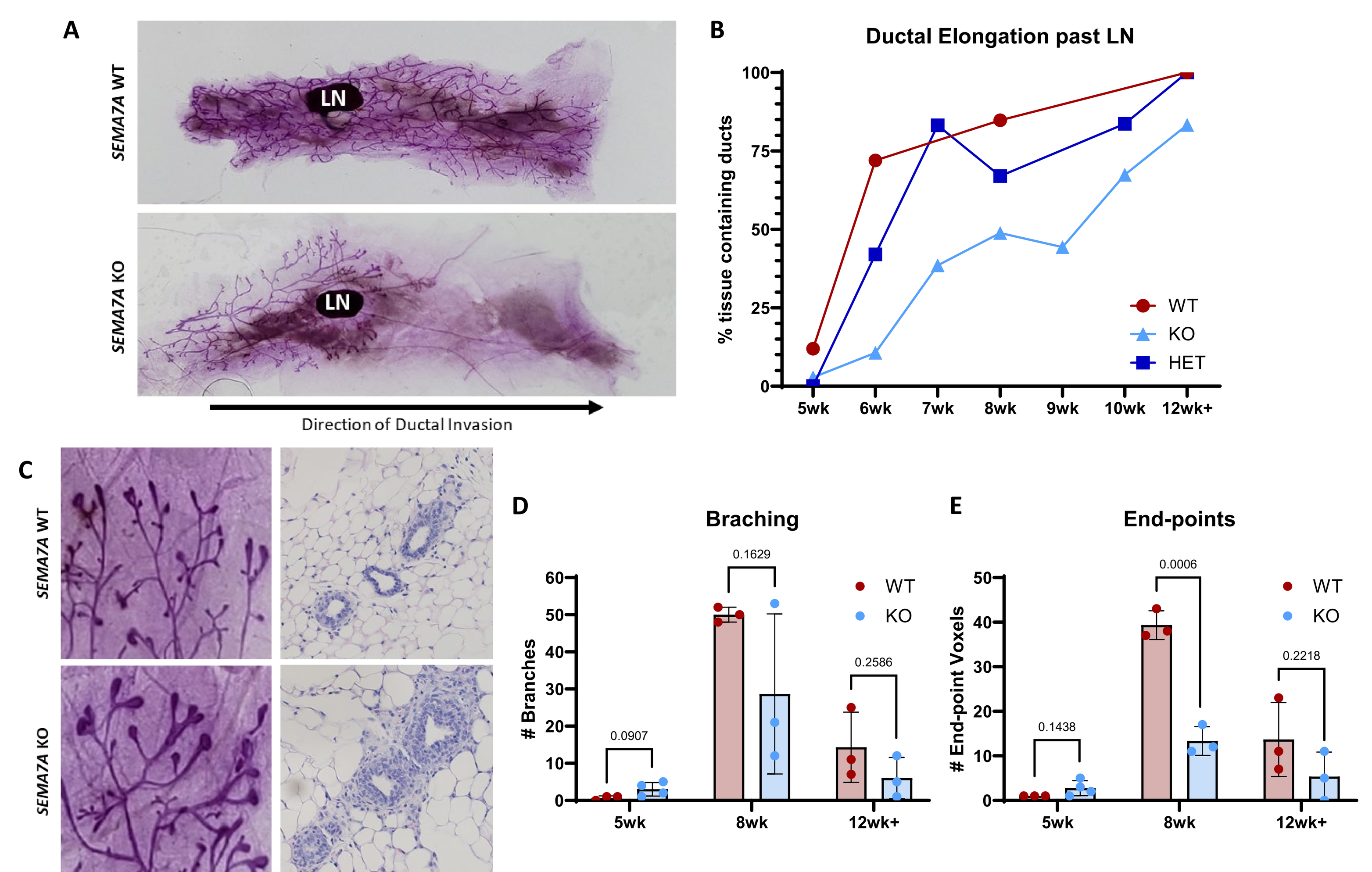


Figure 1: Knocking out *Sema7a* in mice shows reduced ductal elongation during pubertal mammary gland development. (A) Representative mammary gland carmine alum whole mount images from C57BL/6-background *Sema7a* KO mice compared to wild type (WT) at 6 weeks of age. (B) Quantification of ductal elongation at weekly timepoints from 5 weeks of age to 12-13 weeks in WT versus *Sema7a* KO mice. Ductal elongation quantified in millimeters length beyond lymph node, taken as a percentage of millimeter length of mammary gland tissue; WT, KO, HET, n = 3-6. (C) Representative images of (left) mammary gland carmine alum whole mount terminal end buds (TEBs) at 6 weeks of age; (right) H&E images of ducts at 5 weeks of age. (D) Quantification of ductal branching at 5, 8, and 12-13 weeks of age. Branching was quantified using ImageJ AnalyzeSkeleton plug-in with individual thresholds per image; WT, KO, n = 3-4; p-values by two-tailed t-test denoted by numerical value on brackets. (E) Quantification of end-point voxels at 5, 8, and 12-13 weeks of age. End-point voxels represent pixels which constitute a free end of a skeletal branch (duct) using ImageJ AnalyzeSkeleton; WT, KO, n = 3-4; p-values by two-tailed t-test denoted by numerical value on brackets.

Knocking out SEMA7A changes stromal components in the pubertal mammary gland

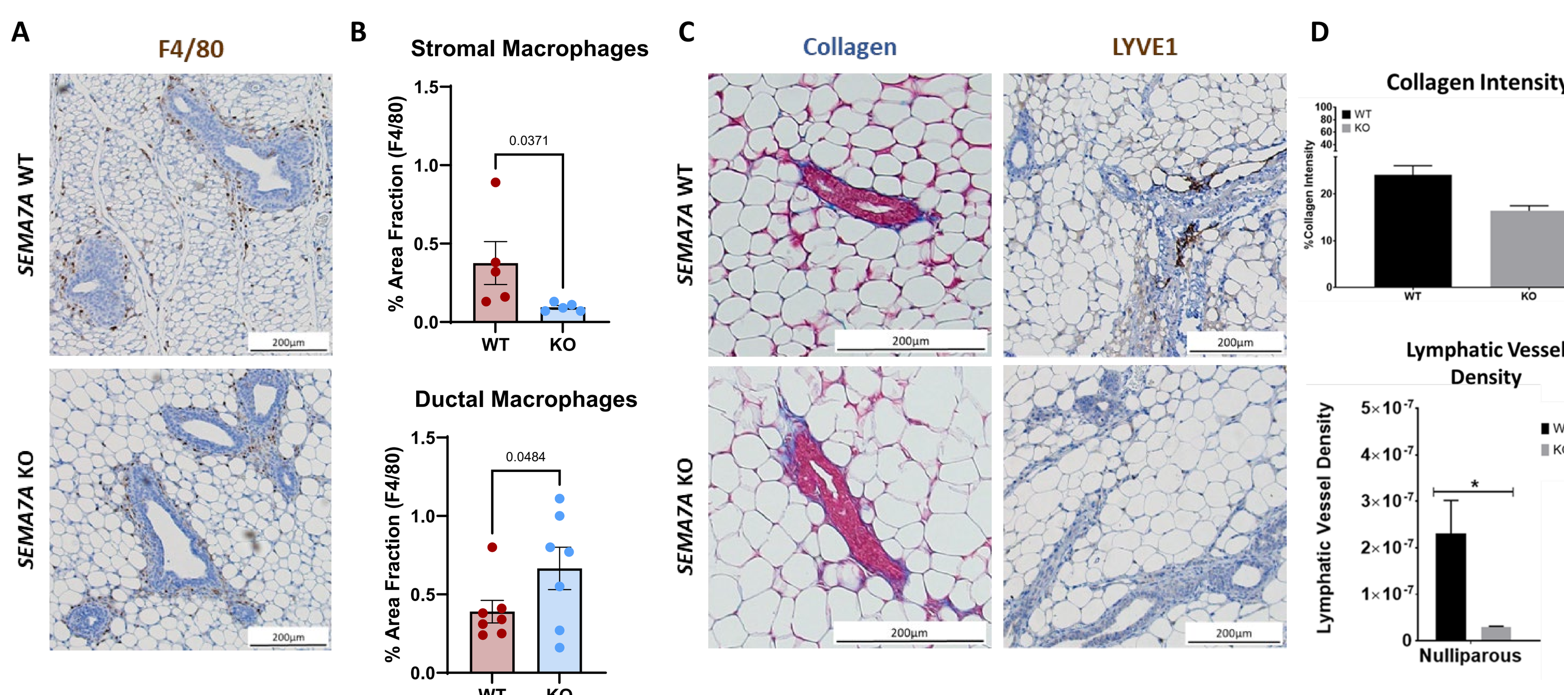


Figure 2: Knocking out *Sema7a* in mice shows reduced stromal macrophages, collagen intensity, and lymphatic vessel density in the pubertal mammary gland. (A) Representative F4/80 IHC images from WT and *Sema7a* KO mice at 5 weeks of age, with F4/80 marking macrophages (brown). (B) Quantification of F4/80 in representative images of the stroma- (top) and duct- (bottom) associated tissue at 5 weeks of age. For the duct, 20x images were taken of the duct and surrounding tissue; for the stroma, 20x images were taken of regions in the mammary fat pad not containing ducts; WT, KO, n=5; p-values by two-tailed t-test denoted by numerical value on backets. (C) Representative images from (left) trichrome stain showing collagen intensity (blue), and (right) IHC for LYVE1 marking lymphatic vessels (brown). (D) Quantification of (top) collagen intensity of trichrome staining at 5 weeks of age, and (bottom) lymphatic vessels density (LVD; number of lymphatics per tissue area (mm^3); WT, KO, n=2; *$p < 0.05$.

SEMA7A alters mRNA expression of macrophage polarization markers

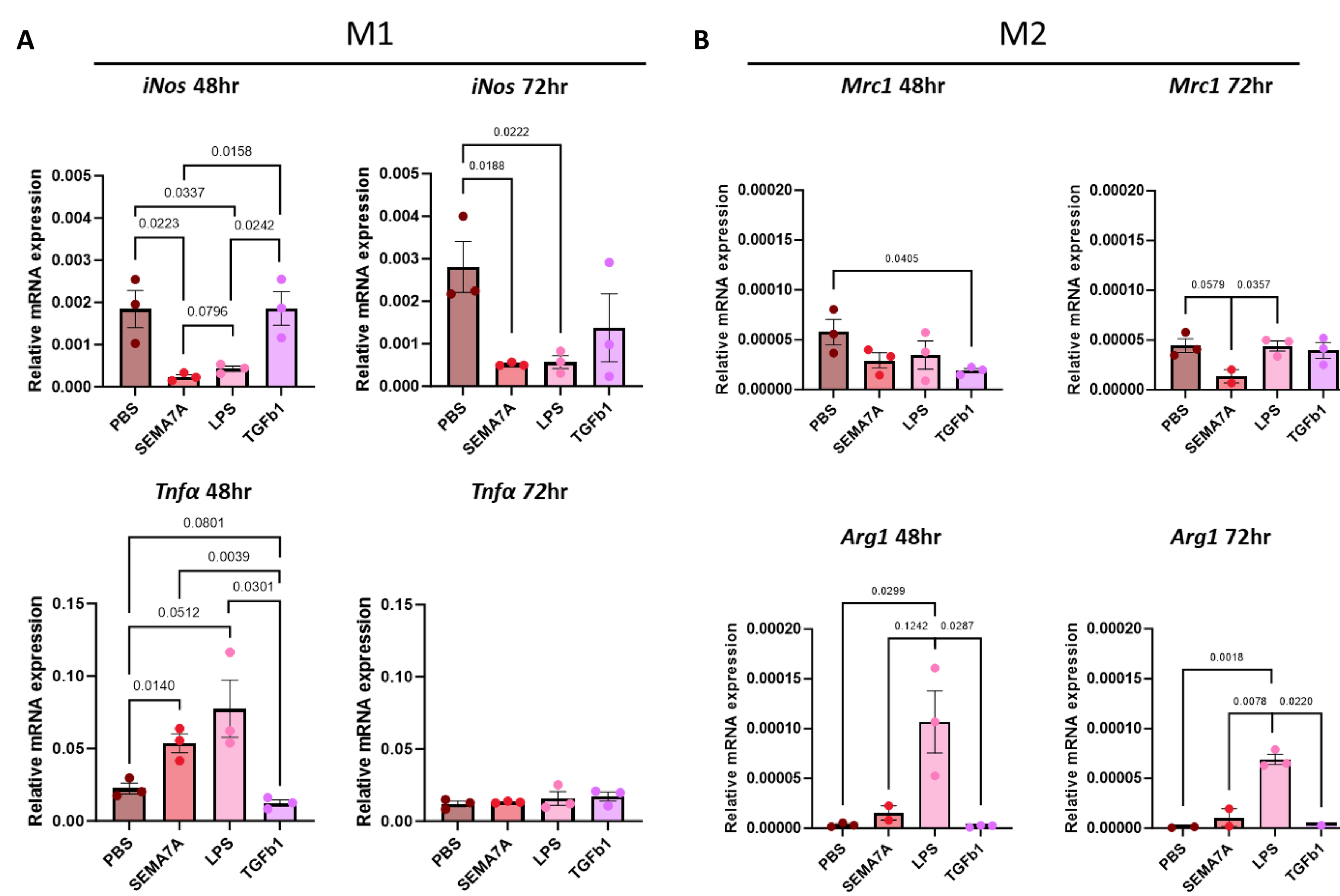


Figure 3: Treatment of RAW265.7 macrophages with SEMA7A promotes a unique mRNA expression pattern. RAW264.7 macrophages were treated with 40µg/mL purified SEMA7A, 100ng/mL lipopolysaccharide (LPS), 20ng/mL TGFβ1, or PBS control. LPS and TGFβ1 were used as controls to stimulate M1 and M2 polarization, respectively. After 48 hours and 72 hours, cell lysates were collected for RNA for cDNA preparation and qPCR analysis. Quantification by Bio-rad CFX Maestro Software. PBS, SEMA7A, LPS, TGFb1, n = 3; p-values by two-tailed t-test denoted by numerical values on brackets. (A) mRNA expression of representative M1 gene expression markers: (top) Inducible nitric oxide synthase (*iNos)* and (bottom) Tumor necrosis factor alpha (*Tnfα)*, relative to *Gapdh*. (right) 48-hour timepoint, (left) 72-hour timepoint. (B) mRNA expression of representative M2 gene expression markers: (top) Mannose-receptor C-type I (*Mrc1;* CD206 protein) and (bottom) Arginase 1 (*Arg1).* (right) 48-hour timepoint, (left) 72-hour timepoint.

SEMA7A alters protein expression of macrophage polarization markers

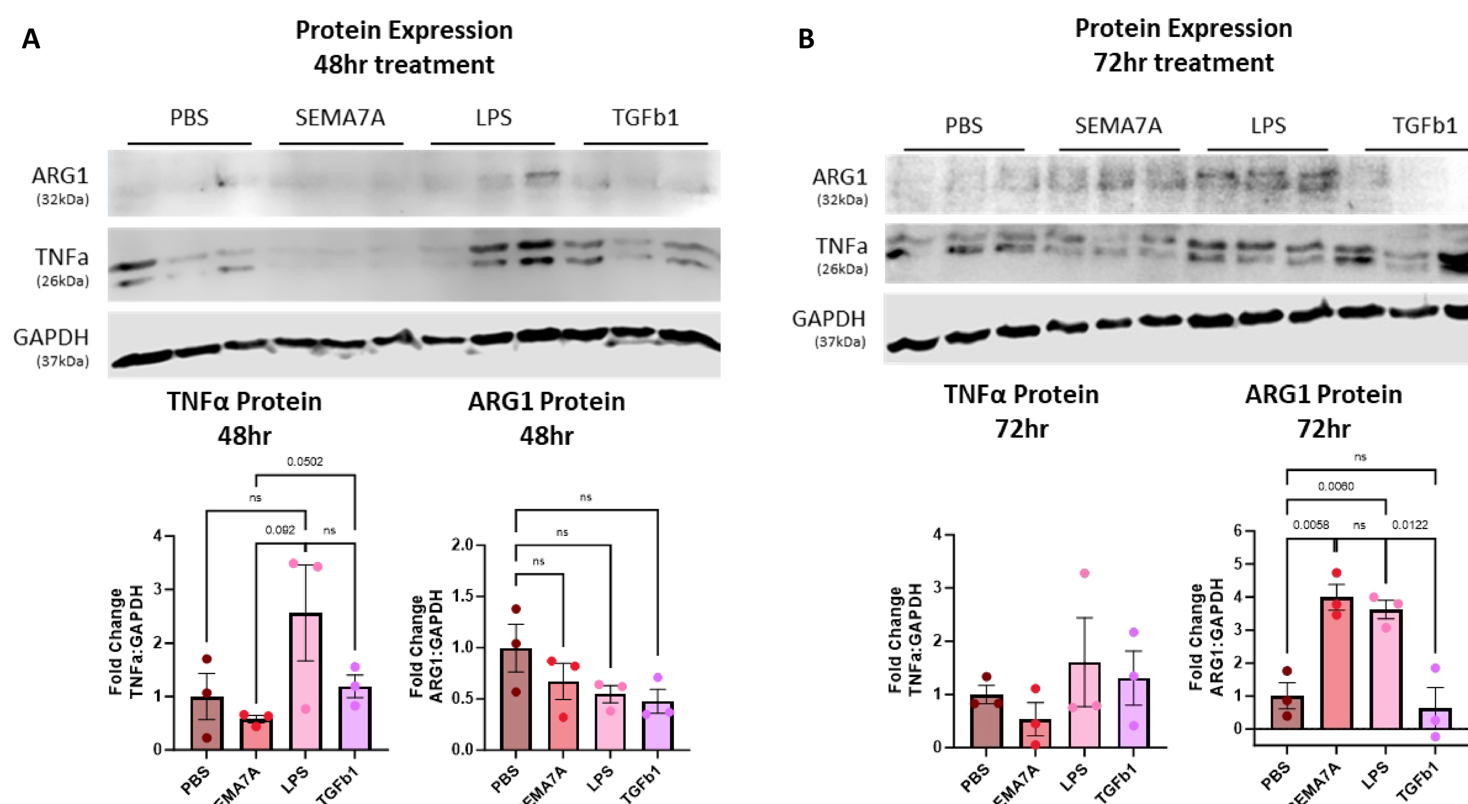


Figure 4: Treatment of RAW265.7 macrophages with SEMA7A promotes a unique protein expression pattern. RAW264.7 macrophages were treated with 40µg/mL purified SEMA7A, 100ng/mL lipopolysaccharide (LPS), 20ng/mL TGFβ1, or PBS control. LPS and TGFβ1 were used as controls to stimulate M1 and M2 polarization, respectively. After 48 hours and 72 hours, cell lysates were collected for protein and western blot analysis. Quantification by Li-Cor Empiria Studio Software. PBS, SEMA7A, LPS, TGFb1, n = 3; p-values by two-tailed t-test denoted by numerical values on brackets. (A) 48-hour timepoint: (top) Representative western blot images of TNFα (M1) and ARG1 (M2) and protein expression. (bottom) Quantification of ARG1 and TNFα expression as fold-change relative to GAPDH. (B) 72-hour timepoint: (top) Representative western blot images of TNFα (M1) and ARG1 (M2) protein expression. (bottom) Quantification of ARG1 and TNFα expression as fold-change relative to GAPDH.

SEMA7A increases macrophage protease secretion and activity

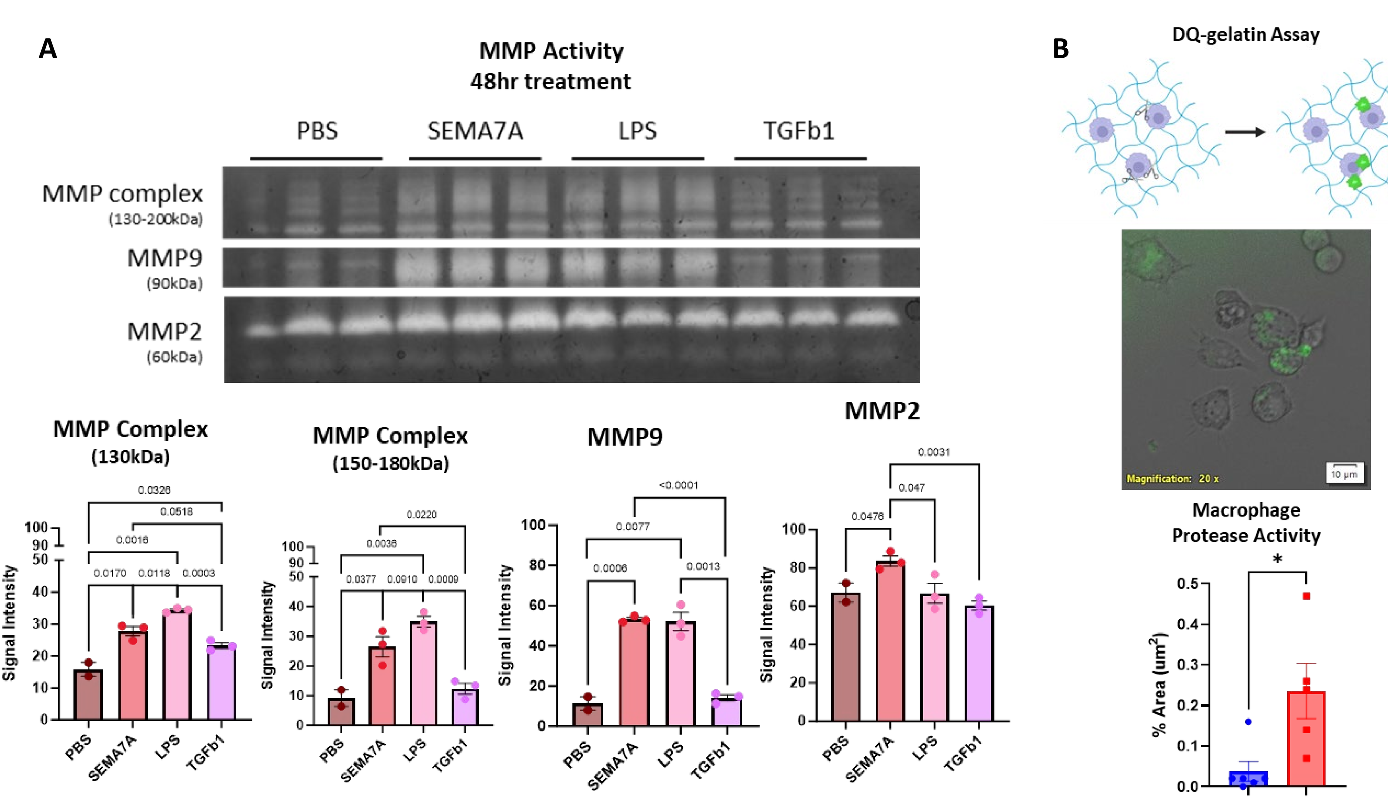


Figure 4: Treatment of RAW265.7 macrophages with SEMA7A promotes protease activity. (A) RAW264.7 macrophages were treated with 40µg/mL purified SEMA7A, 100ng/mL lipopolysaccharide (LPS), 20ng/mL TGFβ1, or PBS control. LPS and TGFβ1 were used as controls to stimulate M1 and M2 polarization, respectively. After 48 hours, conditioned media was collected for zymography analysis. (top) MMP Zymography image. (bottom) Quantification of MMP bands by ImageJ Software. PBS, SEMA7A, LPS, TGFb1, n = 3; p-values by two-tailed t-test denoted by numerical values on brackets. (B) Dye-quenched (DQ)-gelatin assay with RAW264.7 macrophages treated with 40 µg/mL purified SEMA7A or PBS control. (top) Schematic of DQ-gelatin binding to gelatin matrix with green fluorescence denoting protease activity. (middle) Representative IF image of DQ-gelatin protease activity. (bottom) Quantification of green (FITC) fluorescence as a percentage of total area (mm3); n=6, *$p = 0.05$ by two-tailed t-test.

Blocking SEMA7A with a monoclonal antibody decreases DCIS progression and pro-invasive collagen phenotypes

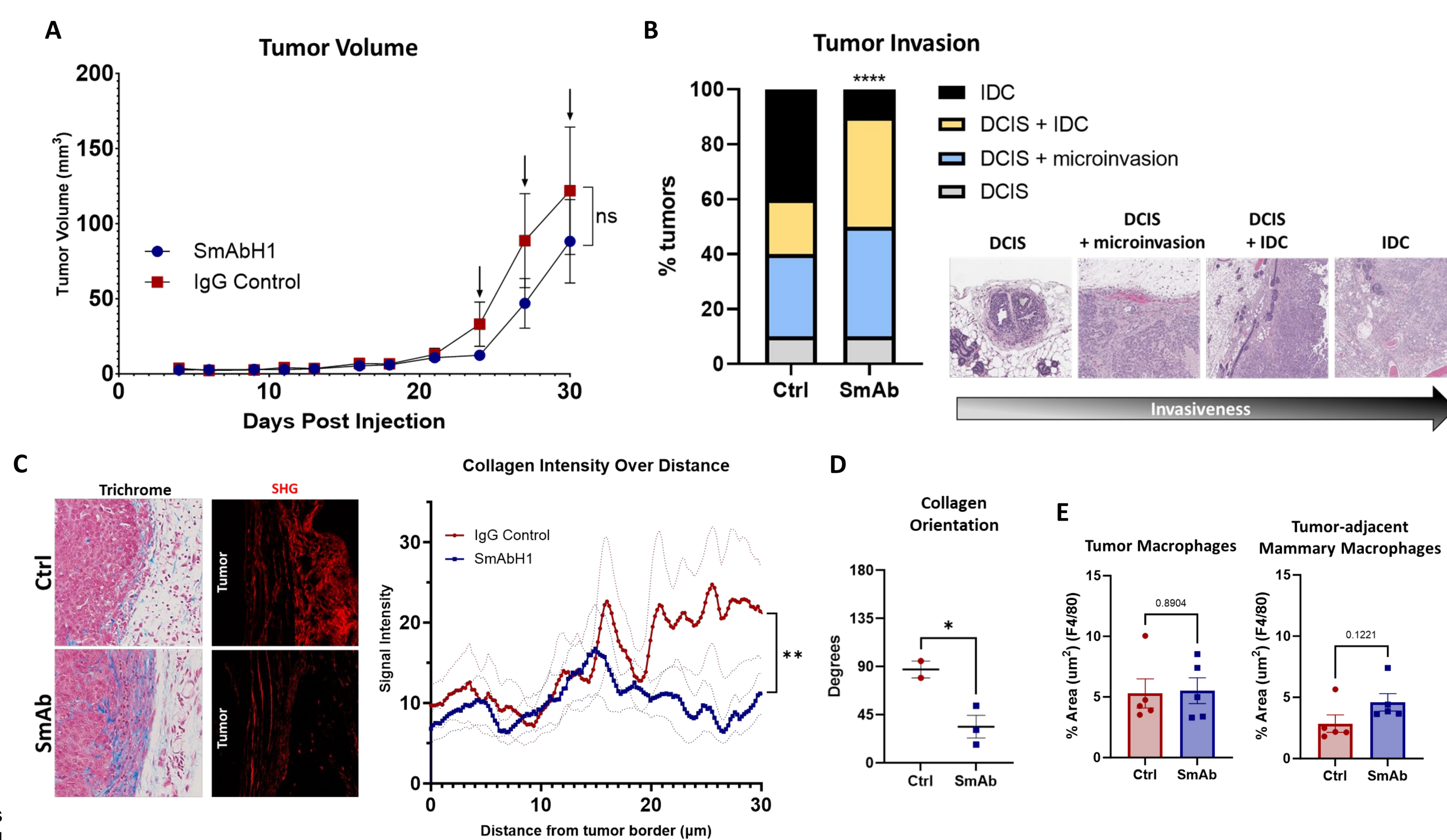
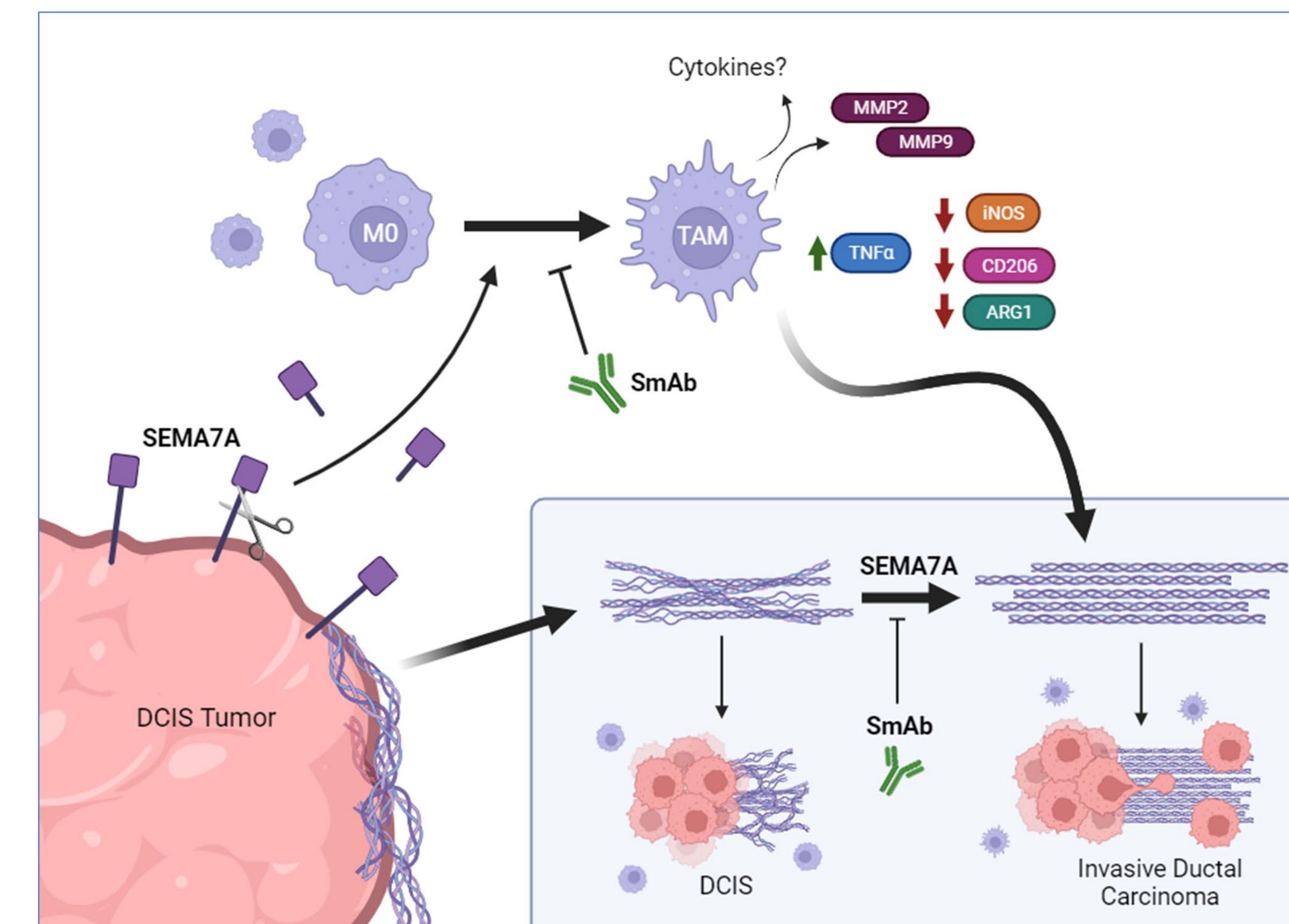


Figure 6: Treatment of murine DCIS tumors with a SEMA7A-blocking antibody decreased DCIS progression and invasion-promoting phenotypes. Female SHO mice were subcutaneously injected with 250,000 MCF10DCIS cells in the left and right #4 mammary fat pad. Mice, n=5 per group, were intraperitoneally injected with 250µg/mouse SEMA7A monoclonal antibody (SmAbH1) or IgG control every 3 days starting 21 days post-tumor cell injection. (A) Tumor volume per group; Ctrl, SmAbH1, n=5, *ns* = not significant by two-tailed t-test. (B) Invasion scores for MCF10DCIS tumors. Invasion was evaluated utilizing a H&E scoring system detailed in bottom right. Ctrl, SmAbH1, n=5; ****$p<0.0001$ by fraction of total analysis. (C) Collagen intensity analysis of MCF10DCIS tumor borders. (left) Representative images of trichrome staining on the tumor border. (middle) Representative images of SHG of the tumor border. (right) Quantification of collagen intensity on SHG imaging of the tumor border; quantification by ImageJ. IgG control, SmAbH1, n=3. (D) Collagen orientation analysis of MCF10DCIS tumor borders. SHG imaging of the tumor border were analyzed utilizing ImageJ collagen orientation plug-in. Ctrl, SmAb, n=3; *$p<0.05$ by two-tailed t-test. (E) Quantification of F4/80 IHC as a percentage of total area (mm^3); Ctrl, SmAb, n=5; p-values by two-tailed t-test denoted by numerical value on brackets. (right) F4/80 quantification in the tumor region only. (left) F4/80 quantification of total mammary gland region and tumor.

Conclusions and Future Directions

- We have shown **a potential delay in pubertal development in *Sema7a* KO mice**, demonstrated through a decrease in ductal length starting at 6 weeks of age (Fig1A-B), sustained macrophage presence around the duct (Fig 2A-B) as well as in the lack of collagen intensity and LVD (Fig 2C-D).
- **Treating macrophages with SEMA7A alters mRNA and protein expression of macrophage polarization markers** (Fig 3-4), most notably TNFα and ARG1. Macrophage "polarization" appears to more closely resemble LPS treatment compared to TGFβ1 treatment.
- **Blocking SEMA7A with a monoclonal antibody decreases DCIS tumor progression in mice** (Fig 5B). These tumors also had decreased collagen intensity from the tumor border (Fig 6C-D) and changes in collagen orientation (Fig 4E) that suggest a less invasive phenotype. Interestingly, we found an increase of macrophages in the tumor-adjacent mammary gland (Fig 6E).

Overall, we have shown that **SEMA7A facilitates cell invasion by altering structural components and macrophage function that assist in cell invasion.** We hypothesize that SEMA7A promotes macrophage accumulation around DCIS tumors, priming macrophages to be tumor-promotional and remodel the collagen around the myoepthelium to assist in DCIS cell invasion. SEMA7A shed from DCIS cells promotes macrophage MMP expression, to remodeling the tumor-associated matrix and assist in the invasive potential of SEMA7A+ DCIS tumors. Blocking SEMA7A signaling may decrease DCIS progression of these tumors. **Our next steps are to characterize cytokine expression in macrophages after SEMA7A treatment and further explore upstream mechanisms of SEMA7A regulation.**



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