Hedgehog signaling stimulates Tenascin C to promote invasion of pancreatic ductal adenocarcinoma cells through Annexin A2

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ABSTRACT
Pancreatic adenocarcinoma (PDA) is characterized by a dense desmoplastic reaction that comprises 60–90% of the tumor, while only 10–40% of the tumor is composed of malignant epithelial cells. This desmoplastic reaction is composed of stromal fibroblast cells, extracellular matrix proteins, and immune cells. Accumulating evidence has suggested that the stromal and epithelial cell compartments interact during the pathogenesis of this disease. Therefore, it is important to identify the signaling pathways responsible for this interaction to better understand the mechanisms by which PDA invades and metastasizes. Here, we show that secreted stromal factors induce invasion of PDA cells. Specifically, hedgehog signaling from the tumor cells induces tenascin C (TnC) secretion from the stromal cells that acts back upon the tumor cells in a paracrine fashion to induce the invasion of PDA cells through its receptor annexin A2 (AnxA2). Therefore, blocking the interaction between TnC and AnxA2 has the potential to prevent liver metastasis in PDA.

KEYWORDS
annexin A2; hedgehog; pancreas cancer; stroma; tenascin C

Pancreatic ductal adenocarcinoma (PDA) is a highly chemotherapy and radiotherapy resistant tumor. Because of the rapid progression of this cancer and the limitations of therapy, the 5-year survival rate for patients with PDA is only 7%. Interestingly, the majority of pancreas tumors are comprised of stromal cells, with only 10–40% of the tumor being PDA cells. Emerging evidence suggests that decreasing the stromal compartment increases the efficacy of some chemotherapeutics, which indicates that the tumor microenvironment may play a role in the progression of PDA.

We used a functional seroproteomic approach that employed pre- and post-vaccination sera from patients vaccinated with a pancreatic cancer vaccine to identify novel PDA tumor antigens. By correlating disease free survival (>3 years) with the presence of particular antibodies in the post-vaccination sera, we identified Annexin A2 (AnxA2), a calcium-dependent phospholipid binding protein, as a new pancreatic tumor antigen. AnxA2 has been shown to be overexpressed in PDA, with expression increasing during the progression from pre-malignant PanINs to PDA.

We further investigated the role of AnxA2 and have shown that phosphorylation of Tyr23 is required for cell surface localization. Following translocation to the cell surface, AnxA2 promotes TGFβ-Rho mediated epithelial-mesenchymal transition (EMT). The role of AnxA2 in invasion and metastasis is further supported through knockdown studies in which knockdown of AnxA2 within the PDA cells suppresses metastasis of these cells to the liver.

Because of the predominance of stroma within the tumor, it is important to understand the signaling pathways within the tumor microenvironment that promote invasion and metastasis of PDA cells in order to develop therapies targeting these processes. Tenascin C (TnC), a known extracellular ligand of AnxA2, is transiently expressed during fetal development but is generally absent in normal adult tissues. However, the expression of TnC increases greatly in pathological conditions, including inflammation and almost all malignant solid tumors. Specifically, TnC has been shown to be localized to the stromal compartment in PDA, with expression increasing from low grade PanINs to PDA, similar to that of AnxA2 expression. However, TnC expression is not correlated with survival, clinical stage, tumor size, nodal status, distant metastasis, or tumor location in patients with PDA. Conversely, the co-overexpression of AnxA2 and TnC in colorectal carcinoma has been shown to be correlated and associated with poor survival.
TnC binding to AnxA2 has been shown to induce the disassembly of focal adhesions and actin stress fibers, which suggests that the interaction between AnxA2 and TnC in cancer promotes cell motility and metastasis. In addition, TnC signaling has been shown to activate ERK/NF-κB signaling to induce resistance to apoptosis and the chemotherapy gemcitabine in PDA. While there is no observed correlation between TnC expression and survival in PDA, recent data suggests that TnC expression does play an important role in predicting a patient’s response to chemotherapy as well as promote the migration and metastasis of PDA. However, the factors that stimulate TnC signaling in the tumor microenvironment of PDA remain unknown.

Published reports suggest that Hedgehog (Hh) signaling is active in stromal cells during pancreatic tumorigenesis, and Hh inhibitors are able to decrease the stromal compartment and metastasis but not primary PDA development. In particular, the de Sauvage group demonstrated a paracrine paradigm for Hh-mediated tumorigenesis, where Hh ligands produced by tumor cells act upon the stromal compartment to support PDA growth and metastasis through an uncharacterized mechanism.

Here, we show that extracellular factors secreted by stromal cells act through AnxA2 to induce invasion of PDA cells. Specifically, we show that TnC secretion from the stromal cells is increased following the induction of Hh signaling, while Hh pathway inhibitors (LDE225, HhAntag) decrease TnC secretion from stromal cells, particularly when co-cultured with PDA cells. In addition, knockdown of TnC from stromal cells decreases invasion of PDA cells both in vitro and in vivo in an Hh-dependent manner. While the role of Hh remains controversial because the depletion of stromal cells through Hh independent and dependent mechanisms in various mouse models results in the promotion of PDA growth and metastasis, we show that Hh signals originating from the PDA cells act upon the stromal compartment to induce the secretion of TnC, a stromal factor that promotes PDA cell invasion through AnxA2. Thus, this study attempted to establish a role of Hh-induced TnC secretion from stromal cells within the tumor microenvironment in promoting the invasion and metastasis of PDA through AnxA2.

Materials and methods

Cell lines and reagents

The Panc02 cell line is an established chemical carcinogen induced mouse PDA cell line that originated from C57Bl/6 mice. Panc02, PDA, mMSCs and C3 cells were maintained in DMEM media (Gibco, catalog no. 11995065) supplemented with 10% FBS (Gibco, catalog no. 10082147), 1% L-glutamine (Gibco, catalog no. 25030081), 1% MEM-NEAA (Gibco, catalog no. 1140076), 1% sodium pyruvate (Gibco, catalog no. 11360070) and 0.5% penicillin/streptomycin (Gibco, catalog no. 15140122). All of the cells were grown in a humidified incubator at 37°C and 10% CO2.

The hedgehog inhibitor NVP-LDE225 was obtained from Novartis (Basel, Switzerland). A 1 mM stock was made by dissolving the inhibitor in DMSO. The inhibitor was further diluted in culture media to a concentration of 1 μM for use in the in vitro assays. The IC50 of LDE225 ranges from 1–24 μM depending on the cell type. However, cell viability is affected at concentrations of approximately 5–10 μM. Therefore, because we wanted to assess the effect of Hh inhibition on other cellular pathways without affecting cell viability, we chose to use 1 μM of LDE225 for our in vitro experiments. This concentration produced sufficient inhibition of Gli-1 by RT-PCR (data not shown). The hedgehog inhibitor HhAntag was obtained from Genentech (San Francisco, CA, USA). A 1 mM stock was made by dissolving the inhibitor in DMSO. The inhibitor was further diluted in culture media to a concentration of 100 μM for use in the in vitro assays. Yauch et al. demonstrated that 10 μM of HhAntag was insufficient to modulate endogenous Hh target genes (Gli-1) in tumor cells. Therefore, we assessed Gli-1 levels following treatment with higher concentrations of HhAntag and found sufficient inhibition of Gli-1 by RT-PCR in our PDA cell line at 100 μM of HhAntag, which did not have an effect on cell viability (data not shown). Recombinant mouse Shh and TnC were both purchased from R&D (rShh: catalog no. 461-SH; rTnC: catalog no. 3358-TC). Recombinant mouse Shh has an ED50 of 0.6–3 μg/mL in most cells. However, 300 ng/mL of rShh demonstrated sufficient activation of Gli-1 in MSCs as determined by RT-PCR (data not shown).

Western blot analysis of Annexin A2

The PDA cells were lysed 48 hours after transfection in 250 mM NaCl, 5 mM EDTA, 50 mM tris (pH 7.4), and 0.5% NP-40 containing protease inhibitors. After lysis, the lysate was spun at 15,000 rpm for 5 min. The supernatant was boiled in SDS sample buffer (Bio-Rad, catalog no. 1610791) containing reducing agents (Bio-Rad, catalog no. 1610792) and was then loaded and electrophoresed on a 4 to 12% bis-tris gel (Bio-Rad, catalog no. 3450124) for 2 hours at 120 V. The gels were transferred onto nitrocellulose membranes at 80 V for 1 hour at 4°C. The membranes were blocked in 5% bovine serum albumin (BSA) overnight at 4°C on a shaker. Annexin A2 primary antibodies (1:1000, Santa cruz biotechnology,
catalog no. sc-9061) were added in 2.5% BSA, and the membranes were incubated at room temperature for 2 hours. The membranes were washed and then incubated with rabbit secondary antibodies conjugated to horseradish peroxidase (1:5000; GE, catalog no. NA934-1ML) for 1 hour at room temperature. The membranes were again washed and then developed using enhanced chemiluminescence reagent (GE, catalog no. RPN2109).

**Transwell invasion assay**

The invasion assay was performed using the Trevigen invasion assay kit according to the manufacturer’s instructions (Trevigen, catalog no. 3455–096-K). In brief, the transwells were previously coated overnight with 1X BME. Then, the cells were plated at 5×10^5 cells per well in triplicate. Invasion was measured 48 hours later by MTT units. Briefly, the cells in the top well were removed and the well was washed with washing buffer provided in the kit. The top well of the transwell was placed in a fresh 96-well plate containing 150 μL of complete cell media and 15 μL of MTT reagent (Roche, catalog no. 11465007001). The plate was returned to the incubator and incubated at 37°C, 10% CO2 for 4 hours in the dark. Following the 4 hour incubation, the top well of the transwell plate was placed in 200 μL of DMSO and incubated at room temperature on a shaker for 20 minutes until the crystals were dissolved. The plate was read at 590 nm and 660 nm. Serum-free media was added to the bottom well of the control. MTT units were adjusted by subtracting the background invasion of the serum-free control from the experimental groups.

**Plasmid transfection, lentiviral infection and RNA interference**

For RNA interference, the cells were seeded in multiple 6-well plates to 80% confluence. For each well, 40 pmol siRNA duplex was transfected with Lipofectamine 2000 (Invitrogen, catalog no. 11668019) in serum-containing, antibiotic-free medium according to the manufacturer’s manual. The cells were harvested 48 hours after transfection and used for invasion analysis. The AnxA2 siRNA was synthesized by Ambion, Inc., and the scramble siRNA was purchased from Ambion (catalog no. AM4611).

To produce lentivirus expressing TnC shRNA, the plasmid with lentiviral constructs was co-transfected with packaging plasmids into 293T cells as previously described.16 Lentiviral supernatant was collected at 48 hours and incubated for 48 hours before the cells were harvested. The lentivirus expressing the mouse TnC hairpin shRNA was obtained from Genecopoeia (catalog no. MSH030372). The cells were sorted by GFP in a FACS cell sorter 48 hours after infection.

**RT-PCR analysis of TnC expression**

RNA was isolated from the cells using the RNeasy Micro kit (Qiagen, catalog no. 74004) and reverse transcribed using the first strand cDNA synthesis kit (Invitrogen, catalog no. 18080051). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was performed with gene-specific fluorescent TaqMan probes (Applied Biosystems, catalog no. 4331182) using an ABI PRISM 7500 Sequence Detection System Instrument and the associated software (Applied Biosystems) according to the manufacturer’s instructions. Each reaction was performed in triplicate. The mouse GAPDH gene (Applied Biosystems, catalog no. 4331182) was used to normalize variations in the quantities of cDNA input.

**TnC ELISA**

TnC secretion was evaluated using the Tenascin C ELISA kit (IBL International, catalog no. 27767) according to the manufacturer’s protocol. The cells were cultured in 0.5% serum containing media for 24 hours prior to evaluating TnC levels in the supernatant. Co-cultured PDA and C3 cells were cultured at a ratio of 1:5.

**Mouse models of PDA**

All of the animal experiments conformed to the guidelines of the Animal Care and Use Committee of the Johns Hopkins University. The animals were maintained in accordance with the guidelines of the American Association of Laboratory Animal Care.

The mouse liver metastasis model was previously established.17,18 This transplanted tumor model allows for in vivo assessment of tumor growth following shRNA knockdown of targeted PDA expressed proteins. In short, the spleens of anesthetized female C57Bl/6 mice ages 8 to 10 weeks were divided into 2 halves, and the halves were clipped. C3 cells with TnC shRNA or C3 cells with control shRNA together with PDA cells were injected into the splenic bed (splenic artery and veins) through one hemispleen followed by a flush of HBSS buffer. Following injection, the splenic vessels draining the injected hemispleen were clipped, and the hemispleen was removed. Finally, the abdominal wall was sutured, and the skin was adapted using wound clips. All
of the mice were followed daily for survival. At necropsy, the livers and injection sites (pancreas/spleen) were removed for histological analysis of metastases.

**Results**

**PDA cell invasion is mediated by secreted stromal factors acting through AnxA2**

To assess the effect of stromal cells on the invasiveness of PDA cells with or without AnxA2 expression, we performed a transwell invasion assay. PDA cells with or without AnxA2 siRNA and stromal cells were plated in a 96-well transwell plate. Supernatant from the stromal cells was added to both PDA and stromal cells. Forty-eight hours later the plate was harvested and invasion was assessed as MTT units. PDA cells treated with supernatant from the stromal cells had the highest levels of invasion (Fig. 1B). The increase in invasion with stromal supernatant treatment was diminished, however, in the PDA AnxA2 siRNA cells (Fig. 1B). This confirms that AnxA2 enhances invasion of PDA cells and that secreted factors from the stroma act through AnxA2 to enhance the invasion of PDA cells.

**Hh signaling regulates the expression of TnC in pancreatic cancer-associated stromal cells in vitro**

Based on the data in the literature demonstrating that Hh signals originating from the tumor cells induces the secretion of several cytokines from the stroma, we wanted to evaluate the particular stromal signals induced by the Hh pathway. Because TnC is known to promote invasion and is a ligand for AnxA2, we hypothesized that TnC secretion is induced by Hh signaling in stromal cells. To confirm our hypothesis, Panc02 cells and mouse MSCs (mMSCs) were plated in single culture and co-culture at \(5 \times 10^6\) and \(1 \times 10^6\) cells per flask, respectively, in mouse MSC media containing 10% FBS. The media was replaced with mouse MSC media containing 0.5% FBS after the cells adhered to the flask to reduce the contribution of serum factors. The following day, the cells were treated with 300 ng/mL rShh or 100 \(\mu\)M HhAntag in 0.5% FBS containing media. Twenty-four and 48 hours following the initiation of treatment, the cells were harvested and the co-cultured cells were sorted into stroma and PDA cells. qRT-PCR analysis was performed on the individual pure populations of tumor and stroma to evaluate the expression of TnC in both cell types.

Recombinant Shh treatment increased the levels of TnC in the stromal cells (Fig. 2A), while treatment with the Hh antagonist HhAntag decreased the levels of TnC in the stromal cells (Fig. 2B). PDA cells secrete very little TnC following co-culture with MSCs, even after stimulation with rShh (Fig. 3A). Specifically, using GFP tagged MSCs, we co-cultured MSC and PDA cells for 24 hours and then treated the cells with 300 ng/mL of rShh for 24 hours. Next, we sorted the cells by GFP status, and used RT-PCR analysis to evaluate the mRNA levels of TnC in both of the cell lines following co-culture. PDA cells (GFP negative) express little TnC after co-culture with MSCs and treatment with rShh, while the MSCs (GFP positive) have an over 50-fold upregulation in the mRNA levels of TnC following co-culture and treatment with rShh (Fig. 3A). Not surprisingly, the level of TnC in the PDA cells was also unaffected by treatment with a hedgehog antagonist (data not shown). This confirms that paracrine Hh signaling results in an upregulation of TnC in the stromal cells (Fig. 3A). Western blot analysis of TnC protein levels was performed on both tumor and stromal cells following the above-mentioned treatments (data not shown). However, the antibodies available

![Figure 1](image-url). Co-culture of MSC and PDA cells leads to increased invasion of PDA cells. A. Transient transfection of AnxA2 targeting siRNA results in an approximately 50% decrease in expression of AnxA2 in PDA cells, as determined by Western blot, compare with cells transfected with scrambled control siRNA. B. PDA cells demonstrate an increased invasion capacity following culture with supernatant from MSCs or co-culture with MSCs. However, this invasive capacity is lost following siRNA knockdown of AnxA2 in the PDA cells.
were inadequate to quantitate expression level differences. Therefore, an ELISA to evaluate TnC secretion from tumor and stromal cells in both single and co-culture was also performed. PDA cells co-cultured with MSCs as well as MSC cells cultured alone were treated in 0.5% serum with 300 ng/mL rShh or 1 μM LDE225 for 24 hours prior to harvesting the cell supernatant for evaluation in the ELISA. We chose to use the hedgehog antagonist LDE225 over HhAntag in this experiment because it appears to be more potent in vitro. However, a more formal comparison of the differences in potency of the inhibitors is required. Additional information pertaining to the in vitro differences between the 2 inhibitors can be found in the methods section. Treatment of mMSCs with rShh or LDE225 had no effect on the levels of TnC secretion. However, TnC secretion was markedly increased when PDA cells and mMSCs were co-cultured. This increase in TnC secretion was further enhanced with rShh treatment and was slightly diminished by LDE225 treatment (Fig. 3B).

**Knockdown of TnC in stromal cells decreases the invasion of PDA cells in an Hh-dependent manner**

To confirm the functional role of enhanced TnC secretion from stromal cells following Hh activation, we performed a shRNA knockdown of TnC in C3 stromal cells. The invasiveness of PDA cells co-cultured with TnC knockdown C3 cells is decreased compared with PDA cells co-cultured with control C3 cells. Recombinant TNC treatment of co-cultured cells does not appear to enhance their invasive capacity above control levels, suggesting that the Hh pathway may already be maximally activated in the co-culture setting. However, inhibition of the Hh pathway by LDE225 decreases the invasiveness of PDA cells co-cultured with either TnC shRNA C3 cells or control C3 cells (Fig. 4). Taken together, this suggests that co-culture of PDA and stromal cells results in Hh-dependent TnC secretion from the stromal cells that acts through AnxA2 to induce invasion of the PDA cells.

**Knockdown of TnC in the stroma demonstrates a trend in survival in a liver metastasis model of PDA**

To evaluate the significance of TnC knockdown in vivo, TnC shRNA knockdown C3 cells or control C3 cells were co-injected with Panc02 cells into the hemi-spleen of C57Bl/6 mice. The mice were monitored for survival. Although not significant, the mice receiving a co-injection of Panc02 cells + TnC shRNA knockdown C3 cells survived slightly longer on average than the mice receiving Panc02 cells + control shRNA C3 cells (Table 1).
One possible reason that the survival was not significantly different is likely because the host mice can contribute stroma to the tumor microenvironment that expressed endogenous TnC, which would compensate the knockdown of TnC expression in the C3 cells. However, this trend does suggest that TnC is important in the invasion of PDA cells in vivo.

**Discussion**

AnxA2 has been implicated in diverse cellular processes including calcium-dependent regulation of endocytosis and exocytosis, focal adhesion dynamics, transcription and translation, cell proliferation, oxidative stress, and apoptosis. AnxA2 functions primarily as an AnxA2-S100A10 heterotrimer (AIIt), which consists of 2 monomeric AnxA2 molecules bridged together by a central dimer of S100A10 proteins. AIIt is able to interact with membrane phospholipids, cytoskeletal F-actin, and extracellular matrix (ECM) components, while monomeric AnxA2 has a low affinity for membrane phospholipids.

Cell surface AIIt has been shown to play a role in ECM degradation. For example, the S100A10 dimer has been shown to bind the pro-protease plasminogen to mediate its' activation into plasin by the plasminogen activators TPA and uPA, which results in downstream proteolytic cascades that result in the degradation of ECM components. Specifically, in vascular endothelium, AIIt activates plasminogen to degrade fibrin-rich blood clots. Further, macrophages and metastatic cancer cells have also been shown to use the plasminogen proteolytic cascade to initiate protease-mediated ECM degradation to enable cell migration.

In addition to its' role in ECM degradation, AIIt can also mediate cell-cell adhesion and vesicle trafficking. In particular, AIIt has been shown to mediate haematopoietic stem cell (HSC) homing and binding to the bone marrow after transplantation. In addition, AIIt has been shown to mediate cell-cell adhesion between breast cancer cells and the microvascular endothelium. Gokhale et al. have shown that AIIt binds vesicles expressing PtdIns(4,5)P2 with high affinity, which suggests a role for AIIt in membrane dynamics, as well as endocytosis and exocytosis. Specifically, AIIt that is associated with PtdIns(4,5)P2-rich membranes interacts with F-actin in a calcium-dependent manner at regions of active membrane-cytoskeleton remodeling including cell-cell junction formation, vesicular transport, cell polarization, and cell migration. Because the formation of actin networks are regulated by small GTPases, such as Rho, Rac1, and Cdc42, it is not surprising that AnxA2 could be purified from complexes containing Rac1 at actin-rich cell-cell contacts. Data demonstrating the association between AnxA2, actin, and the hyaluronan receptor CD44 suggests that AnxA2 can act as a scaffold protein to link the actin cytoskeleton and various membrane microdomains or factors involved in actin remodeling. Finally, AIIt has been shown to function in adherens junction formation in both endothelial cells and epithelial cells by associating with epithelial E-cadherin and endothelial VE-cadherin.

In addition to AnxA2, multiple cell surface receptors for TnC have been identified. Interestingly, because TnC is a large, extracellular matrix molecule of approximately 300 kDa that contains 4 individual domains, it is believed that each domain binds a different type of extracellular receptor for a distinct function. For example, the tenascin assembly domain can form intermolecular hydrophobic interactions and disulfide bridges with various molecules. The epidermal growth factor-like repeats of TnC act as low affinity ligands for the EGF-receptor (EGFR), which results in activation of mitogen-activated protein kinase (MAPK) and phospholipase-C (PLC)-γ signaling. Additionally, the fibronectin type III-like repeats (FNIII) interact with integrins, aggrecans, and perlecans, and also binds to members of the platelet-
derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor-β (TGFβ) families. Finally, the fibrinogen-like globule (FBG) binds to integrins, receptor-type tyrosine-protein phosphatase zeta (PTPRz), and molecules that activate Toll-like receptor-4 (TLR4) signaling. Due to the various domains of TnC binding diverse receptors, TnC can activate diverse processes such as mitogenic processes, cell migration, cell attachment, cell spreading, focal adhesion, cell survival, and pro-inflammatory cytokine synthesis. Specifically, integrins such as α2β1, α7β1, α8β1, α9β1, ovβ3, and αdβ6 have been shown to bind to the FNIII domain of TnC and promote cell attachment. While AnxA2 also binds to the FNIII domain of TnC, AnxA2 binding to TnC results in disassembly of focal adhesion and actin stress fibers to promote cell detachment and motility. TnC has also been shown to bind protein receptor tyrosine phosphatase β (PTPβ). Although the exact effect of this binding is unknown, it is not believed to be related to cell adhesion. Additionally, the EGF-like domain of TnC binds to the epidermal growth factor receptor (EGFR) to promote EGFR phosphorylation. Therefore, because of the wide range of functions driven by TnC, the effect of TnC binding to a receptor likely depends on the type of receptor as well as the cell type expressing that receptor. Of note, there are also known ‘long’ and ‘short’ splice variants of TnC that can bind various different receptors and have different functional consequences both during embryonic development and carcinogenesis.

It is well-established that TnC, specifically the FNIII1A-D region of TnC, binding to AnxA2 in pancreatic cancer increases the intracellular phosphorylation of the protein kinase B signaling cascade proteins, which include PI3k, Akt, IKKα, and NF-κB. Although the effects of FNIII1A-D has not been assessed in the context of promoting cell survival, proliferation, or growth, since these proteins are known to promote these processes, it is plausible that FNIII1A-D binding to AnxA2 can promote metastatic growth of pancreatic cancer to the liver by promoting the survival and proliferation of these cells once they reach the liver. Specifically, other signaling pathways acting through AnxA2 can promote cell invasion and motility of PDA cells. However, once the cells reach the liver, it is possible that the FNIII1A-D domain of TnC on liver stromal cells binds to AnxA2 on pancreatic cancer cells to induce the phosphorylation of PI3k, Akt, and MAPK to promote cell survival, growth, and proliferation of PDA cells in this new environment. This signaling, thus, could establish a tumor microenvironment that favors cell survival in the 'foreign' organ to allow metastatic lesions to develop and grow. Therefore, blocking this interaction has the potential to prevent metastatic lesions from forming in the liver. Interestingly, breast cancer cells have been shown to produce TnC, which enhances the expression of stem cell signaling components, such as musashi homolog 1 (MSI1) and leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), in the tumor microenvironment. In this study, breast cancer cell-derived TnC was essential for metastatic outgrowth until the stroma in the tumor microenvironment could take over as the source of TnC. While in our study, the stromal cells served as the source of TnC, the findings of Oskarsson et al. highlight the important role of TnC as an extracellular matrix component of the metastatic niche and support the development of therapies targeting TnC to prevent metastatic lesions from developing and growing in various types of cancers.

Based upon the results of this study, future studies are warranted to address the precise role of TnC in the tumor microenvironment. Specifically, TnC knockout mice or TnC targeting therapies should be used to determine if the interaction between TnC and AnxA2 is critical for metastatic homing and colonization. Our study was limited because the mice used in our study expressed TnC and were able to recruit TnC expressing stromal fibroblasts to the liver to potentially aid in colonization. Therefore, using a TnC knockout mouse or an antibody to inhibit TnC would enable us to identify if TnC is essential for colonization at metastatic sites and if suppression of TnC expression/activity would inhibit micrometastases formation. Additionally, because TnC has been shown to play a role in maintaining the viability of the stem cell niche, it would be important to address if the viability of the stem cell niche could be suppressed by therapeutically targeting TnC to eliminate or prevent the formation of micrometastases and whether such a role is executed through AnxA2. Therefore, future studies should focus on elucidating the exact mechanism by which TnC promotes metastatic formation through AnxA2 in order to determine if TnC-targeting therapies would be clinically useful in preventing or treating metastasis.

**Abbreviations**

- AnxA2: Annexin A2
- ALIt: nxA2-S100A10 heterotetrramer
- BME: basal membrane extract
- DMEM: Dulbecco's Modified Eagle Medium
- DMSO: dimethyl sulfoxide
- ECM: extracellular matrix
- EGFR: epidermal growth factor receptor
- ELISA: enzyme-linked immunosorbent assay
EMT epithelial to mesenchymal transition
FACS fluorescence activated cell sorting
FBG fibrinogen-like globe
FBS fetal bovine serum
FGF fibroblast growth factor
FNIII fibronectin-like III domain
HBSS Hank’s balanced salt solution
Hh Hedgehog
HhAntag a hedgehog antagonist produced by Genentech
HSC haematopoietic stem cells
LGR5 leucine-rich repeat-containing G protein-coupled receptor 5
MAPK mitogen-activated protein kinase
mMSC mouse mesenchymal stem cells
MEM-NEAA minimum essential medium-non-essential amino acids
MSI1 musashi homolog 1
MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NVP-LDE225 a hedgehog antagonist produced by Novartis
PanINs pancreatic intraepithelial neoplasms
PDA pancreatic ductal adenocarcinoma
PDGF platelet-derived growth factor
PLC phospholipase C
PRTPβ protein receptor tyrosine phosphatase β
PTPRζ receptor-type tyrosine-protein phosphatase zeta
Shh sonic hedgehog
TGFβ transforming growth factor-β
TLR4 Toll-like receptor 4
TnC Tenascin C

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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