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Microenvironmental Factors Drive Tenascin C and Src Cooperation to Promote Invadopodia Formation in Ewing Sarcoma

Allegra G. Hawkins*, Claire M. Julian†, Sonja Konzen*, Sydney Treichel*, Elizabeth R. Lawlor* and Kelly M. Bailey†

*Department of Pediatrics, Rogel Cancer Center, University of Michigan, Ann Arbor, MI, USA, 48109; †Department of Pediatrics, Division of Hematology/Oncology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA 15224

Abstract

Ewing sarcoma is a bone tumor most commonly diagnosed in adolescents and young adults. Survival for patients with recurrent or metastatic Ewing sarcoma is dismal and there is a dire need to better understand the mechanisms of cell metastasis specific to this disease. Our recent work demonstrated that microenvironmental stress leads to increased Ewing sarcoma cell invasion through Src activation. Additionally, we have shown that the matricellular protein tenascin C (TNC) promotes metastasis in Ewing sarcoma. A major role of both TNC and Src is mediation of cell–cell and cell-matrix interactions resulting in changes in cell motility, invasion, and adhesion. However, it remains largely unknown, if and how, TNC and Src are linked in these processes. We hypothesized that TNC is a positive regulator of invadopodia formation in Ewing sarcoma through its ability to activate Src. We demonstrate here that both tumor cell endogenous and exogenous TNC can enhance Src activation and invadopodia formation in Ewing sarcoma. We found that microenvironmental stress upregulates *TNC* expression and this is dampened with application of the Src inhibitor dasatinib, suggesting that TNC expression and Src activation cooperate to promote the invasive phenotype. This work reports the impact of stress-induced TNC expression on enhancing cell invadopodia formation, provides evidence for a feed forward loop between TNC and Src to promote cell metastatic behavior, and highlights a pathway by which microenvironment-driven TNC expression could be therapeutically targeted in Ewing sarcoma.

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Introduction

Ewing sarcoma is a bone or soft tissue tumor that most commonly presents in adolescents. Patients who present with primary, localized disease have a survival rate of greater than 70% following intensive, compressed interval chemotherapy and radiation and/or surgery [1]. However, patients who present with metastatic disease, or who experience relapse, have survival rates of <20% [2]. Although much has been learned about the biology of Ewing sarcoma tumorigenesis, mostly related to the driver EWS/ETS fusion [3], comparatively little is known about the cellular mechanisms that drive Ewing sarcoma metastatic progression. At present, the same multi-agent chemotherapy is used for patients who present with both localized and metastatic disease, and intensification of neoadjuvant and adjuvant therapy has proven to be of no benefit for patients with progressive disease [4]. Gaining a better understanding of the biologic drivers of metastasis is necessary for the development of more specific and effective therapeutic targets to treat this cancer.

We have recently reported that changes in the local tumor microenvironment can induce phenotypic changes in Ewing sarcoma cells to states that support migration, invasion, and metastatic engraftment [5–8]. In particular, both microenvironmental stresses (hypoxia, nutrient deprivation, growth constraints) and activation of canonical Wnt signaling can induce Ewing cells to activate pro-metastatic gene signaling programs. Once active, these metastatic

Abbreviations: ECM, extra-cellular matrix; TME, tumor microenvironment; TNC, tenascin-C

Address all correspondence to: Kelly M. Bailey, MD, PhD, 4401 Penn Ave, Rangos Research Building 5th floor Rm 5132, Pittsburgh, PA 15224. E-mail:Received 3 April 2019; Revised 16 August 2019; Accepted 20 August 2019

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programs trigger Ewing cell actin-cytoskeleton changes including the formation of invadopodia, activation of CXCR4, Src and Rac/Cdc42, and secretion of extra-cellular matrix (ECM) proteins, such as tenascin C (TNC) and collagen I [5–8].

Activation of Src kinase occurs in many cancers and affects cellular processes including, but not limited to, adhesion, migration, invasion, and cell morphology [9]. Once Src is activated, its primary role is to mediate cell–cell and cell-matrix interactions. As a key regulator of signaling pathways aberrantly activated in cancers, Src is a prime candidate for development of therapeutic targets [10]. In Ewing sarcoma, we have reported that phosphorylation of Src at the Tyr418/419 activating site is enhanced upon nutrient deprivation and hypoxia. The activation of Src results in increased formation of the actin-rich protrusions known as invadopodia, and the formation of invadopodia can be pharmacologically blocked by the Src-kinase inhibitor dasatinib [5].

In addition to invadopodia, another contributor to the metastatic phenotype in Ewing sarcoma is the matricellular protein TNC [6]. We have shown that activation of Wnt/beta-catenin induces transcription and secretion of TNC resulting in increased lung engraftment in Ewing tumor xenografts [6,7]. TNC is a matricellular protein found in the ECM that binds to both fibronectin and collagen fibrils in the tumor microenvironment (TME) as well as integrins on the cell surface [11]. Interestingly, TNC has also been reported in other solid tumors to be associated with enhanced Src phosphorylation [12]. Given our prior work independently demonstrating the importance of TNC in promoting Ewing sarcoma cell metastatic behavior both in vitro and in vivo [6,7] and Src in promoting stress-induced invadopodia formation [5], we sought to determine whether Src and TNC cooperate to alter invadopodia formation in Ewing sarcoma. It is not known whether changes in TNC expression occur when Ewing sarcoma cells face microenvironmental stress such as nutrient deprivation and hypoxia; additionally, the impact of Src on cell endogenous TNC expression is unknown. In the current study, we investigate the impact of TNC and Src on Ewing cell invadopodia formation by determining the ability of both endogenous (tumor cell derived) and exogenous (from the tumor microenvironment) TNC to activate Src and conversely, the impact of Src activation on TNC expression.

Materials and Methods

Cell Culture

Ewing sarcoma cell lines A673 and TC32 were maintained in RPMI 1640 media (Gibco) supplemented with 10% FBS (Atlas Biologicals) and 2 mmol/L-glutamine (Life Technologies). CHLA10 was maintained in IMDM media (Fisher Scientific) supplemented with 20% FBS, 2 mmol/L-glutamine and 1X Insulin-Transferrin-Selenium (Gibco). A673 was obtained from ATCC. CHLA10 and TC32 were obtained from the Children's Oncology Group (COG) cell bank (cogcell.org). All cell lines undergo STR profiling (to ensure cell line identity) and mycoplasma testing (to ensure cultures are free of mycoplasma infection) at routine intervals. For hypoxia experiments, cells were cultured in 1% O2 as previously described [5].

Reagents

Dasatinib was a gift from the Leopold lab (Ann Arbor, MI) and used at a concentration of 50 nM as previously described [5]. Recombinant tenascin C was purchased from R&D Systems (Cat # 3358TC, Minneapolis, MN). Recombinant Wnt3a was purchased from R&D Systems (Cat # 5036-WN-010, Minneapolis, MN) and used at a concentration of 100 ng/ml.

Lentiviral Transduction

Lentiviral production and transduction was performed as described previously [5] and Sigma TRCN0000230788 (shTNC) was used. Transduced cells were selected in puromycin (2 μg/ml).

Gene Expression Analysis

Total RNA was extracted from cells using Quick-RNA MicroPrep (Zymo Research) and cDNA was generated using iScript (Bio-Rad). Quantitative real-time PCR (qRT-PCR) was performed using universal SYBR-Green Supermix (Bio-Rad) for designed primers. Analysis was performed in triplicate using the Light-Cycler 480 System (Roche Applied Science) and average Cp values were normalized relative to the housekeeping gene HPRT. The following primers were used:

TNC forward: 5′GCAGCTCCACACTCCAGGTA3′,
TNC reverse: 5′TTCAGCAGAATTGGGGATTT3′,
HPRT forward: 5′TGACACTGGCAAAACAATGCA3′, and.
HPRT reverse: 5′GGTCCTTTTCACCAGCAAGCT3′.

Immunocytochemistry

Cells were plated directly onto gelatin coated chamber slides and treated under conditions of serum starvation (0% FBS in media) and/or hypoxia (1% oxygen using a BioSpherix Incubator). After 48 hours of serum starvation and/or hypoxia, slides were fixed with 4% paraformaldehyde, permeabilized with .05% Triton-X, and blocked with 5% goat serum for 30 minutes. Slides were incubated with mouse anti-tenascin C (Cat # T2551 Sigma Aldrich) at a concentration of 1:100 in 5% goat serum overnight before incubation with a goat anti-mouse AlexaFluor 555 secondary antibody (Cat # A-21422 Life Technologies). Nuclei were stained with DAPI. Images were visualized using an Olympus IX83 inverted microscope. Number of total cells and number of TNC positive cells were counted in at least four high-power field images using ImageJ.

Western Blotting

Conditioned media from tumor cells was collected and protein was concentrated using 3-kDa cutoff Amicon Ultra Centrifugal Filter Units (Fisher). Protein concentration was measured using the DC Protein Assay (Bio-Rad). Western blot analysis was performed using the Bio-Rad Mini-PROTEAN Tetra System. See reference for TNC western blot protocol [7]. Antibodies were obtained from the following sources: pSrc (Tyr418 Cat # 44-660G Invitrogen/Thermo Fisher, Waltham, MA), Src (Cat # 710449 Thermo, Rockford, IL), vinculin (Cat # 6896 R&D, Minneapolis, MN) and tenascin C (Cat # T2551 Sigma Aldrich). Density of all protein of interest bands were normalized to either vinculin or total protein and fold change relative to vehicle or control was determined and is displayed under each western blot.

Invadopodia/Matrix Degradation Assay

Oregon Green 488 labeled gelatin coated chamber slides were made as previously described [5]. Ewing tumor cells were cultured on the slides and following completion of the experiment, the cells/slides

were fixed with paraformaldehyde, permeabilized with 0.1% Triton X, and quenched with 50 mM NH_4Cl prior to blocking in 5% goat serum in PBS. Slides were incubated with anti-cortactin (EMD Millipore 16–229, Temecula, CA) at 1:500 and DAPI at 1:10,000 in 5% goat serum in PBS overnight. Slides were then imaged using a Zeiss LSM 510 confocal microscope. Total degradation was quantified using ImageJ as described in [13] in 5 high power field images per chamber. Degradation was normalized to the number of cells in each field and averaged over all 5 images.

Statistics

All *P*-values were calculated using Student's *t* test. * indicates $P < .05$ and ** indicates $P < .005$.

Results

Tenascin C Promotes Three-Dimensional Growth of Ewing Sarcoma Cells

We have previously demonstrated that tumor cell-derived tenascin C (TNC) is necessary for metastatic lung engraftment in Ewing sarcoma [6]. TNC secreted into the tumor microenvironment (TME), by either tumor cells or stromal cells, can alter tumor: TME crosstalk through signaling to tumor cells, stromal cells, and components of the extra-cellular matrix. High expression and secretion of TNC by both stromal cells and tumor cells has been reported in a variety of cancers including, but not limited to, breast, colorectal, and prostate [14–16]. TNC plays a variety of roles in dictating how cells interact with their local microenvironment, most prominently being its adhesive and counter-adhesive effects on cells [17,18]. Specifically, TNC can bind and activate integrins, resulting in decreased or increased cell adhesion, dependent on the context [11,18]. Whether or not TNC contributes to adhesive or counter-adhesive effects in Ewing sarcoma is unknown. To determine the impact of exogenous TNC on Ewing sarcoma cell adhesion, A673, CHLA10 and TC32 cells were seeded onto TNC or vehicle control treated plates. Following a 24-hour incubation, cell morphology was assessed. As shown in Figure 1, a striking difference in cellular morphology is noted between the cells placed on a control versus TNC-coated surface in all three Ewing cell lines. Whereas Ewing cells readily formed classic cell monolayers on control plates, under TNC-coated plate conditions, Ewing cells favor cell–cell interactions and growth in three-dimensional spheres (Figure 1, *bottom panels*). From this data, we conclude that Ewing cell contact with extracellular TNC promotes three-dimensional cell interactions and growth.

Tumor Cell Endogenous and Exogenous Tenascin C Enhances Src Phosphorylation in Ewing Sarcoma

Shifts in cell-matrix and cell–cell interactions, such as those noted in Ewing cells upon seeding onto a TNC coated surface, occur through dynamic engagement of integrins and altered signaling through focal adhesion complexes [11]. TNC is able to bind to integrin receptors, thereby enhancing activation of focal adhesion kinase and Src [12,19,20]. In some adult cancers, such as breast cancer, TNC has been implicated in contributing to Src phosphorylation [12]. Src activity is largely mediated through the phosphorylation of the positive regulatory Tyr418/419 [21]. Tyr418/419 is phosphorylated upon the interaction of Src with plasma membrane-bound molecular partners, such as integrins (resulting in cell cytoskeletal reorganization) or receptor tyrosine kinases (resulting in altered cell migration and proliferation) [9,22]. Given our prior data demonstrating the importance of both TNC and Src in metastatic Ewing cell biology, we next wanted to determine if TNC influences Src activation in Ewing sarcoma. We hypothesized that TNC present in the TME from either tumor [7] or stromal cell secretion, may activate Src kinase in Ewing tumor cells. To test this hypothesis, TC32, A673 and CHLA10 cells were treated with recombinant TNC or vehicle for 24 hours and cell lysates were analyzed by western blot for phosphorylation of Src on tyrosine (Y) 418. TNC application to Ewing sarcoma cells results in a significant increase in Src activation (Figure 2*A*). Little change in total Src expression was noted under these conditions.

Having demonstrated that TNC present in the microenvironment can enhance Src activation in Ewing sarcoma, we next turned to examine the impact of tumor cell endogenous production of TNC on Src Y418 phosphorylation. We have previously reported that Ewing sarcoma cells exposed to serum starvation and hypoxia show increased activation of Src [5]. We thus hypothesized that stress dependent activation of Src could be dependent, at least in part, on endogenous expression of TNC. Knockdown of *TNC* using two short hairpins

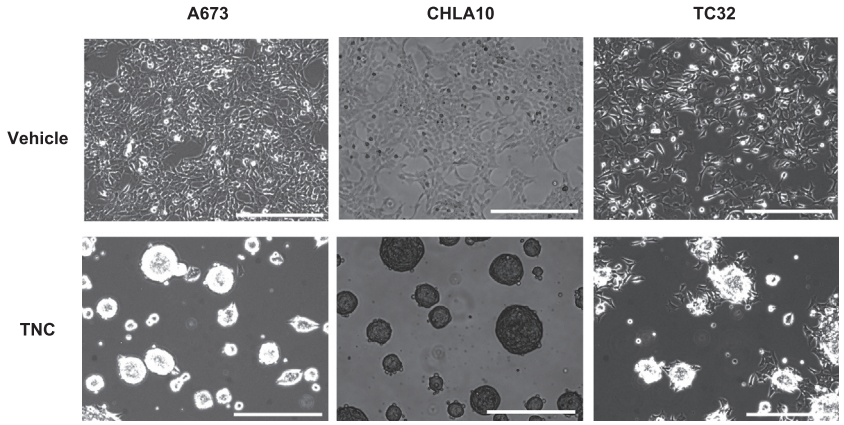

Figure 1. Tenascin C promotes three-dimensional growth of Ewing sarcoma cells. Plates were coated with 5 μg/ml recombinant TNC or PBS prior to addition of Ewing sarcoma cells. Pictures of cells were taken 24 hours after seeding. Cells cultured in the presence of TNC form three-dimensional spheroids. Pictures were taken at 10x magnification and bars are equivalent to 100 μM.

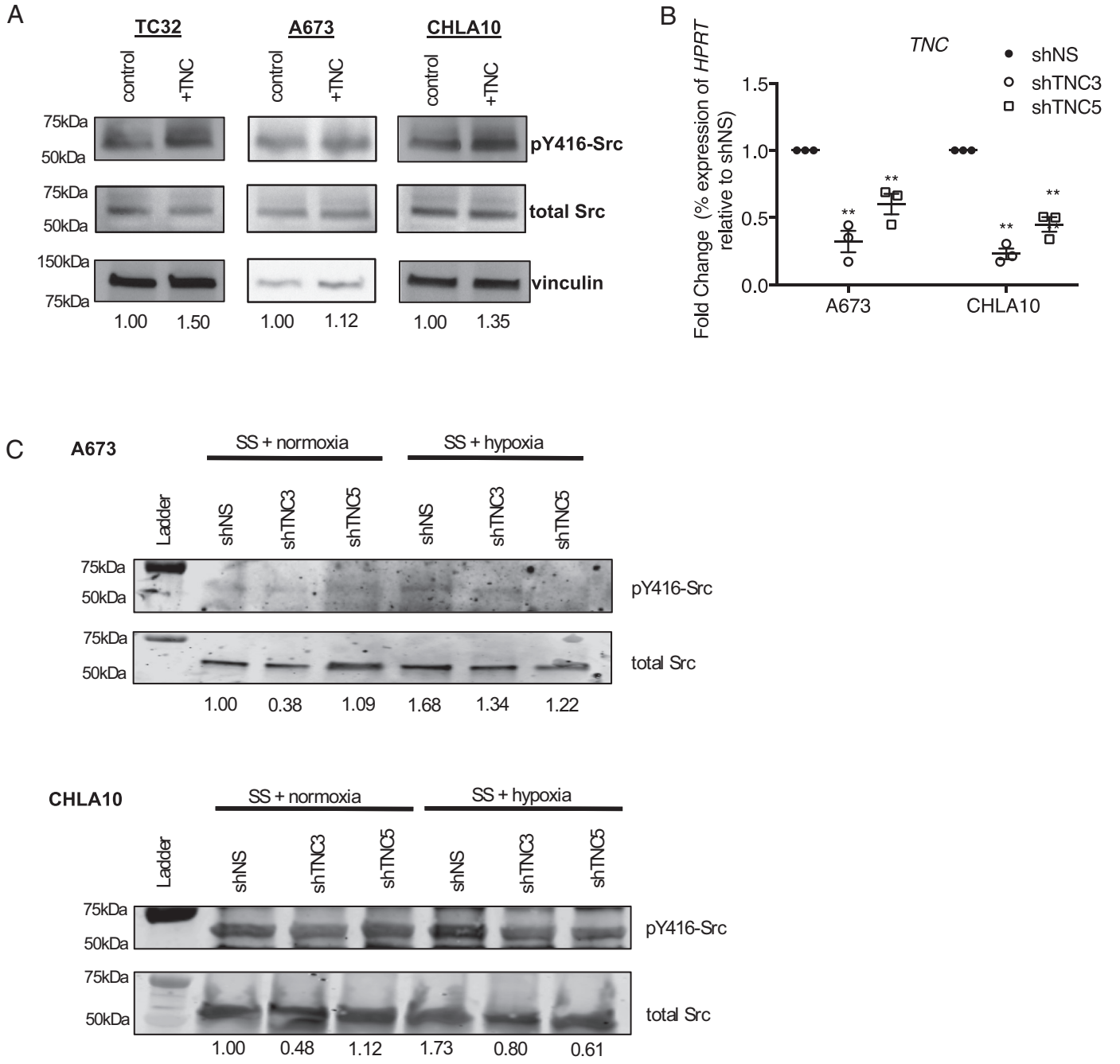

Figure 2. Cell intrinsic and extrinsic tenascin C enhances Src phosphorylation in Ewing sarcoma. A, TC32, A673 and CHLA10 Ewing sarcoma cells were treated with 3 micro-Molar recombinant tenascin C (TNC) or vehicle control for 24 hours. Lysates were subjected to western blot analysis for p-Y416 Src, total Src and vinculin. B, A673 and CHLA10 cells were stably transduced with lentivirus containing non-silencing hairpin (shNS) or two different TNC hairpins (shTNC3 and shTNC5). Knockdown was confirmed via qRT-PCR for *TNC*. C, p-Src and Src expression were measured via western blot in both A673 (top panels) and CHLA10 (bottom panels) cells transduced with control (shNS) or TNC knockdown (shTNC3 and shTNC5) and in the presence of serum starvation (SS), normoxia or hypoxia.

(shTNC3 and shTNC5) was first confirmed by qRT-PCR (Figure 2*B*) and loss of TNC protein expression was confirmed using ICC (Supplemental Figure 1). Next, control and TNC knockdown Ewing cells were subjected to stress conditions including serum deprivation and hypoxia. The results show that lowering TNC expression in tumor cells reduces baseline levels of Src activation and prohibits stress-induced activation of Src kinase (Figure 2*C*). Together, these data demonstrate that both endogenous and exogenous TNC contribute to Src activation in Ewing sarcoma.

Tenascin C Promotes Stress-Mediated Formation of Invadopodia

Invadopodia are actin-rich cell structures that serve as lead points for cell mediated degradation of ECM [23]. Accumulation of the actin assembly protein, cortactin, and matrix metalloproteases

(MMPs) are key factors in mediating formation and function of the invadopodia structure [23,24]. Formation of invadopodia results in the coordination of cell motility with ECM degradation. Src phosphorylation at tyrosine 418/419 is a positive regulator in generating these structures in many cancers, including Ewing sarcoma (as we have previously reported) [5]. Additionally, *TNC* expression in cancer is associated with a more metastatic phenotype [6], specifically through increased expression at the invasive front of many solid tumors [25].

Thus, having established that TNC promotes Src activation in Ewing sarcoma, we next sought to determine if TNC impacts invadopodia formation. We first questioned whether exogenous TNC present in the TME would alter invadopodia formation and subsequent matrix degradation under conditions of stress. To address this, Ewing sarcoma cells were seeded onto Oregon green 488 labeled gelatin-coated chamber slides as previously described [5] and subjected to serum deprivation in addition to application of vehicle or recombinant TNC for 24 hours. Cells/slides were fixed and invadopodia/areas of matrix degradation were imaged (Figure 3*A*) and quantified (Figure 3*B*). TNC application resulted in a statistically significant increase in matrix degradation compared to vehicle treated cells. These data support our hypothesis that increased TNC in the

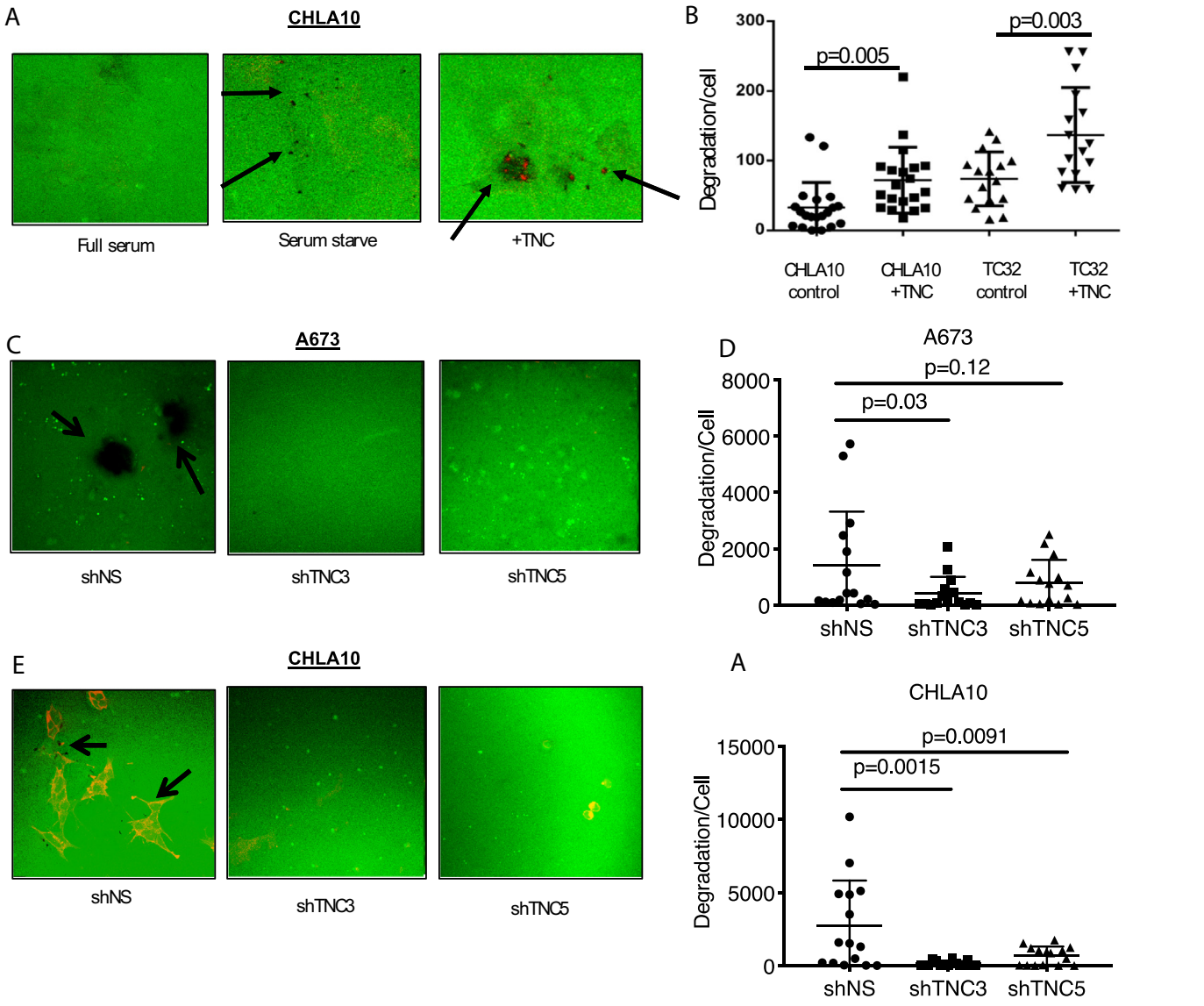

Figure 3. Tenascin C promotes stress-mediated formation of invadopodia. Ewing cells were cultured in 0% FBS-containing culture media for 24-hours on Oregon Green 488 labeled gelatin coated chamber slides in the presence of vehicle control or 3 micro-Molar TNC. A. Representative confocal microscopy images of invadopodia in CHLA10 cells under no stress (left), serum starved treated with vehicle (middle), or serum starved treated with 3 micro-Molar TNC (right) B. Degradation was quantified using ImageJ analysis to determine degradation/cell number for each image. Quantification of area of degradation per cell in both CHLA10 and TC32 Ewing sarcoma cells treated with vehicle control or 3 micro-Molar TNC**.** A673 (C) and CHLA10 (E) were cultured for 24 hours on Oregon Green 488 labeled gelatin coated chamberslides. Cells were incubated on slides in serum starved and hypoxia conditions. Representative high-power field images are shown. D&F. Degradation was quantified as in (B). green = Oregon green 488 gelatin, red = 566 cortactin, black = area of matrix degradation. Arrows indicate areas of invadopodia formation as indicated by punctate cortactin and gelatin degradation. Experiments were performed in triplicate and *P*-values were determined using Student's *t*-tests.

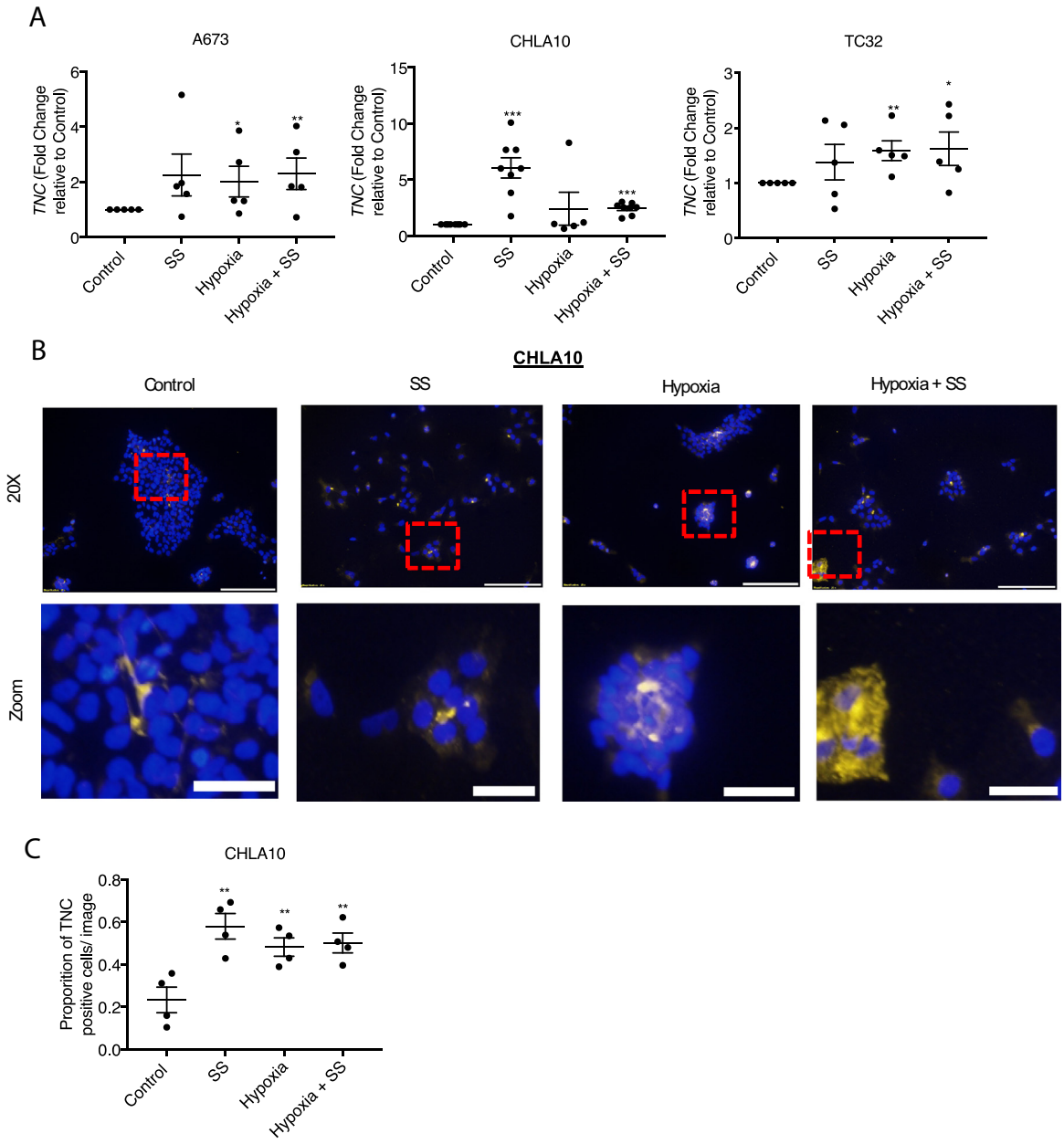

Figure 4. Stress induces tenascin C expression in Ewing sarcoma. A, A673, CHLA10, and TC32 cells were cultured with full serum (10% FBS for A673, 20% FBS for CHLA10) in normoxia (control), 0% serum in normoxia (SS), full serum in hypoxia (Hypoxia), and 0% serum in hypoxia (Hypoxia + SS). Cells were collected after 24 hours in stress and TNC expression was measured using qRT-PCR. B, Representative images of CHLA10 cells cultured in the same conditions as described in (A) for 48 hours before immunocytochemistry staining for TNC, indicated in yellow. Top row is images taken at magnification 20X (Scale bar = 20 μM), bottom row is zoomed in image of top row (Scale bar = 5 μM). Red squares indicate zoomed in areas. C, Proportion of cells that are positive for TNC in high power-field images in CHLA10. *P*-values were determined using Student's *t*-tests and * indicates $P < .05$, ** indicates $P < .005$.

microenvironment enhances the invasive phenotype by promoting invadopodia formation under conditions of stress.

Given our findings that the presence of TNC in the Ewing sarcoma cell microenvironment enhances invadopodia formation (Figure 3*A*), and that TNC directly impacts Src activation (Figure 2), we next sought to determine if formation of stress-induced invadopodia requires endogenous TNC. Control and *TNC* knockdown Ewing cells were cultured on Oregon green 488 labeled gelatin-coated chamber slides and exposed to stress conditions (serum deprivation and hypoxia). As shown in Figure 3, *C–F* **and** Supplemental Figure 2, knockdown of TNC in Ewing sarcoma cells significantly impeded their ability to induce invadopodia and to degrade matrix under conditions of stress. Based on these findings, we conclude that tumor cell endogenous production of TNC contributes to stress-dependent formation of invadopodia and that exogenous TNC can further enhance invadopodia formation and matrix degradation. These data provide evidence for a mechanistic link between Src, TNC, and the invasive Ewing sarcoma cell phenotype.

Stress Induces Tenascin C Expression in Ewing Sarcoma

Having demonstrated the importance of TNC to stress-induced Src activation and invadopodia formation, we next asked: Does stress enhance TNC expression in Ewing sarcoma cells? We have previously shown that expression of TNC in Ewing sarcoma cells is heterogeneous and dynamic, and that it can be induced by canonical Wnt signaling [6]. To test whether stress also induces dynamic changes in Ewing cell TNC expression, A673, CHLA10, and TC32 cells were exposed to conditions of no stress (full serum plus normoxia (control)), single stress (no serum plus normoxia (SS) or full serum plus hypoxia (Hypoxia)) or dual stress (no serum plus hypoxia (Hypoxia + SS)) for 24 hours and *TNC* was measured using qRT-PCR. Both single and dual stress conditions resulted in increased *TNC* expression in all cell lines (Figure 4*A*). Notably, although the degree of observed responses varied among the cell lines in the different conditions, a statistically significant increase in *TNC* was reproducibly detected under conditions of dual stress (serum starvation and hypoxia). In parallel, immunocytochemistry detection of TNC showed that various single and dual stresses resulted in a statistically significant increase in TNC protein (Figure 4, *B*–*C*). Based on these results, we conclude that exposure of Ewing cells to hypoxia and serum deprivation increases *TN*C transcript and protein levels, suggesting that stress induced invadopodia formation occurs, at least in part, because of stress-induced expression of *TNC*.

The Src Inhibitor Dasatinib Decreases Stress-Induced Tenascin C Expression in Ewing Sarcoma

Having shown that stress increases both Src activation and TNC expression in Ewing cells, and that stress-induced invadopodia formation is abrogated by either *TNC* knockdown or Src inhibition, we next questioned whether Src activation can contribute to TNC expression. We first sought to determine the impact of Src on TNC expression in the absence of stress. Ewing cells were exposed to low nanomolar doses of dasatinib and *TNC* expression was measured. Across three different cell lines, we observed a decrease in basal *TNC* expression in dasatinib treated cells as compared with vehicle treated cells (Figure 5*A*). This suggests that Src contributes to expression of *TNC* by Ewing sarcoma cells under basal, unstressed conditions.

Next, to determine if Src activation also contributes to stress-induced *TNC* expression, cells were cultured in control or dual-stress conditions with and without dasatinib treatment. We observed an increase in *TNC* in cells treated with hypoxia and no serum, similar to observations seen in (Figure 4), and this induction was blocked by the addition of dasatinib (Figure 5*B*). Based on these findings, we conclude that both basal and stress-induced *TNC* expression are positively influenced by Src activation.

Dasatinib Inhibits Wnt-Induced Tenascin C Expression in Ewing Sarcoma

We have previously reported that activation of the Wnt/beta-catenin pathway results in increased expression and secretion of TNC [6,7]. Src kinase has also been implicated in playing an important role in Wnt/beta-catenin driven cancers [26–28]. Therefore, we hypothesized that in

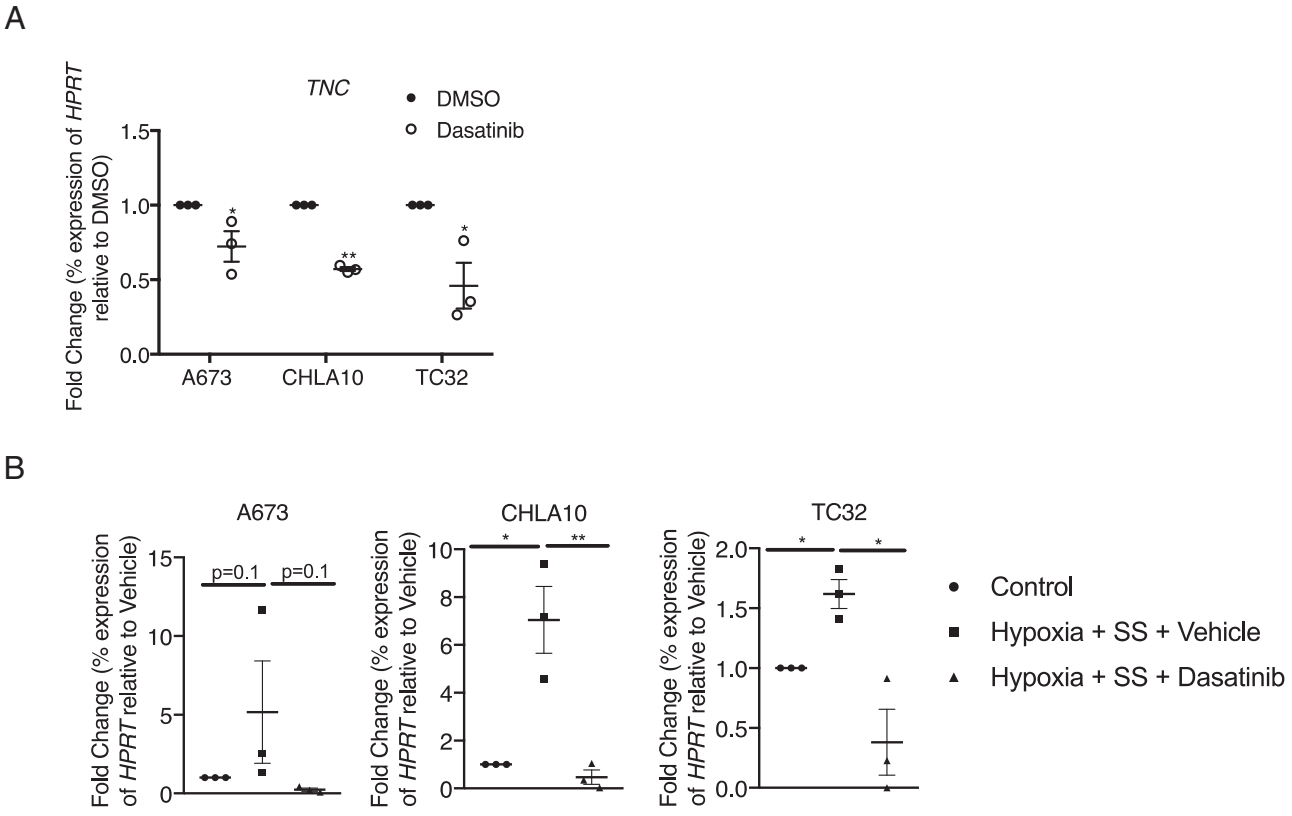

Figure 5. The Src inhibitor dasatinib decreases tenascin C expression in Ewing sarcoma. A, A673, CHLA10, and TC32 cells were treated with either vehicle control (DMSO) or 50 μM dasatinib for 24 hours prior to collecting to determine *TNC* expression via qRT-PCR. B, Cells were cultured for 24 hours in full serum plus normoxia (control), 0% serum plus hypoxia (Hypoxia + SS + Vehicle), and 0% serum plus hypoxia in the presence of dasatinib (Hypoxia + SS + Dasatinib). *TNC* expression was measured using qRT-PCR. Experiments were performed in triplicate and *P*-values were determined using Student's *t*-tests and * indicates $P < .05$, ** indicates $P < .005$.

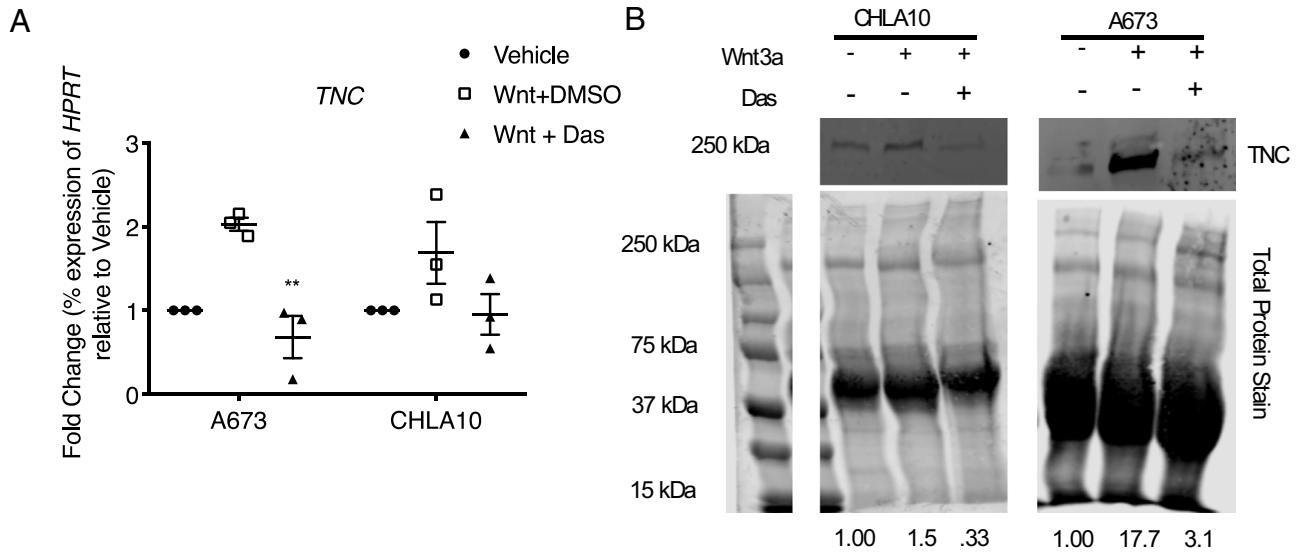

Figure 6. Dasatinib inhibits Wnt-induced Tenascin C expression in Ewing sarcoma. A, Cells were treated with Wnt3a (100 ng/ml) or vehicle (PBS) for 24 hours with or without 50 μM dasatinib. *TNC* expression was measured using qRT-PCR. B, Cells were cultured for 5 days with or without Wnt3a treatment once a day and protein in the conditioned media was collected. 2 days prior to media collection, cells were serum starved and treated with either vehicle or 50 μM dasatinib once a day. Western blot for TNC secretion was performed and showed an increase in TNC secretion with Wnt3a alone and a decrease in TNC secretion with both Wnt3a and dasatinib.

addition to stress-induced TNC expression, Wnt-dependent expression of TNC may also be dependent on Src activation in Ewing sarcoma. In preparation to test this hypothesis, Ewing cells were treated with Wnt or vehicle control in the presence or absence of dasatinib. RNA/corresponding cDNA from these conditions were analyzed by RT-PCR and as shown in both A673 and CHLA10 cell lines, exposure of cells to dasatinib blocked Wnt dependent induction of *TNC* (Figure 6*A*). Next, to we sought to determine whether Wnt-induced TNC secretion is also impacted by dasatinib. Ewing cells were treated with Wnt3a for 3 days and then dasatinib was added for 48 hours prior to collecting secreted protein. Consistent with our prior studies, activation of Wnt/beta-catenin led to increased TNC secretion. We found that the addition of dasatinib

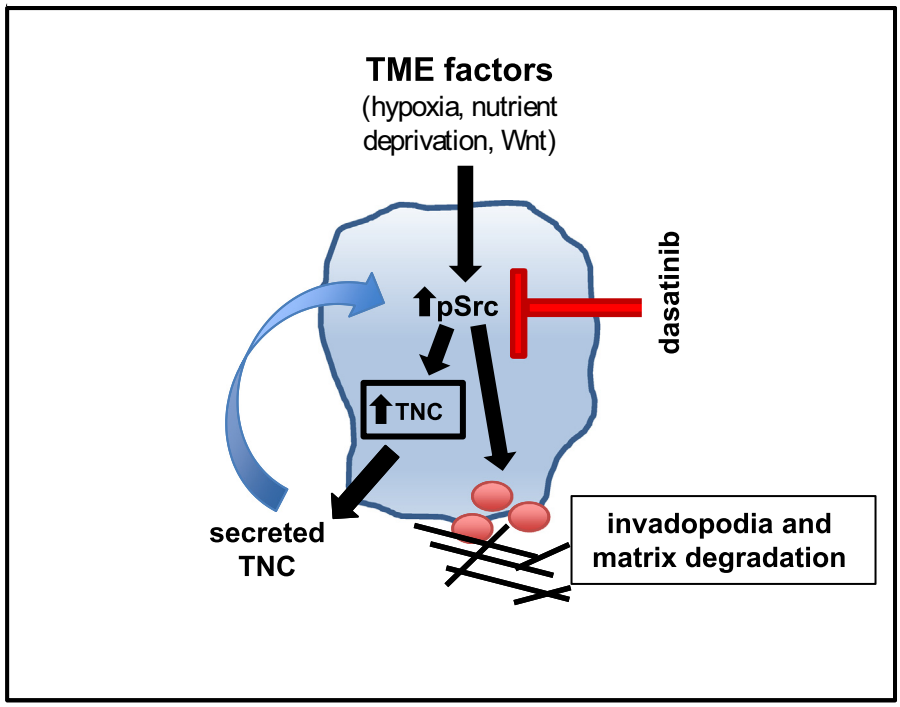

Figure 7. Visual overview. Both tumor microenvironmental (TME) stress and Wnt can upregulate TNC expression in Ewing sarcoma cells. TME-mediated upregulation of TNC and can be blocked by application of dasatinib. Cell exposure to or expression of TNC results in increased invasive potential via Src activation and invadopodia formation.

abrogated this effect (Figure 6*B*). These data show that induction of *TNC*/TNC by canonical Wnt signaling is also Src-dependent.

In sum, these data suggest that in Ewing sarcoma cells, TNC and Src are linked by a feed forward loop in which microenvironmental stimuli, including stress and exposure to canonical Wnt ligands, induce Src-dependent up regulation of *TNC*. Activation of Src and up-regulation of *TNC* leads to increased secretion of tumor cell-derived TNC. Secretion of tumor cell endogenous TNC and exogeneous TNC can feedback to further activate Src and promote formation of invadopodia and matrix degradation (Figure 7). Exogeneous TNC cannot rescue invadopodia formation in dasatinib treated cells, suggesting that the impact of TNC on invadopodia requires active Src (Supplemental Figure 3). Together, these data provide evidence for cooperation between microenvironmental stimuli, TNC, and Src in driving an invasive phenotype in Ewing sarcoma cells.

Discussion

Along with our prior published data, work presented here demonstrates the importance of tumor microenvironmental stress, Src phosphorylation, and TNC on promoting a more metastatic Ewing sarcoma cell phenotype [5–7]. In particular, the current studies lead us to propose a model in which stress induces activation of Src kinase, and in turn, expression and secretion of TNC is increased. TNC in the tumor microenvironment then promotes a feed forward loop in which TNC increases Src activation, thus enhancing Ewing tumor cell invadopodia formation (Figure 7). To our knowledge, this is the first report linking stress/p-Src/TNC in dictating the invasive potential of Ewing sarcoma. In addition, the results demonstrate that in the absence of stress, canonical Wnt ligands in the tumor microenvironment can utilize the same Src/TNC/invadopodia feed-forward loop.

TNC has been shown to play both adhesive and counter adhesive roles in various microenvironments and is able to dictate cell–cell and cell-matrix interactions [11,18]. TNC is most predominantly studied for its ability to bind to fibronectin domains, thus blocking the ability of cells to bind to a fibronectin matrix and favoring cell–cell interactions over cell-matrix interactions [18]. Ewing sarcoma cells have been shown to secrete high amounts of fibronectin [7], implying that TNC in the TME could bind to the fibronectin secreted by Ewing cells to alter Ewing cell adhesion to the ECM. Our studies are the first to begin evaluating the role of TNC on mediating Ewing sarcoma cell–cell interactions and reveal that high levels of TNC lead to favoring of cell–cell interactions and growth of cell spheroids rather than 2D cell spreading. This finding reveals a novel link between TNC and signaling pathways involved in mediating cell–cell interactions and cell morphology, such as Src, in Ewing sarcoma.

We have demonstrated that reduced tumor cell TNC expression results in a decreased efficiency of Ewing cells to form invadopodia, while provision of exogenous TNC enhances invadopodia formation in Ewing tumor cells. TNC has been shown to promote an invasive phenotype in a neuroendocrine tumor model [29] as well as promote a metastatic phenotype through increased migration and activation of the epithelial to mesenchymal transition [12]. Despite this, TNC has yet to be described to contribute to formation of the invasive structures known as invadopodia in cancer. We have now shown that TNC promotes the Ewing cell the invasive phenotype through activation of Src and subsequent invadopodia formation.

Our studies have also revealed that enhanced TNC expression is one of the critical components of the Ewing cell response to tumor microenvironmental-stress. TNC expression is controlled by mechanical stress [30,31]. The activation of TNC through changes in external tensile stress has been heavily described in wound repair, but very little is understood about what activates TNC in a tumor environment. Here, we describe the regulation of TNC expression by microenvironmental stresses [30,31] often found in within tumors, such as nutrient deprivation and hypoxia. Such stress-induced TNC could explain the high expression of TNC in the stroma of some solid tumors. Expression of TNC in bone tumors, such as Ewing sarcoma, could also be attributed to the abundance of Wnt ligand in the bone microenvironment, given our data demonstrating that the Src inhibitor dasatinib blocked Wnt-mediated upregulation of TNC. Thus, inhibition of Src may be a potential therapeutic avenue by which to manipulate microenvironmentally driven TNC expression. Future studies will explore the efficacy of Src inhibition to reduce TNC expression in vivo and will explore combinations to optimally reduce Ewing tumor cell metastatic potential.

Conclusions

The expression and function of TNC are important to consider when devising new treatment schema to test for the prevention or treatment of metastatic Ewing sarcoma, as activation of Wnt/beta-catenin, exposure to microenvironmental stress, and activation of Src all converge to induce expression of TNC. These studies have shown that TNC is a clear hub in pathways that result in pro-metastatic cellular phenotypes. Future studies will begin to exploit TNC as a therapeutic target to reduce metastatic potential in Ewing sarcoma.

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