Wnt/Beta-Catenin Signaling and the Tumor Microenvironment in Ewing Sarcoma Progression

by

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Dedication

To all of the strong and resilient kids who have been affected by cancer. All of this work is because of you in hopes of increasing survival rates and decreasing long term toxicities in pediatric cancer patients.

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TME	tumor microenvironment
EMT	epithelial to mesenchymal transition
ECM	extracellular matrix
MSC	mesenchymal stem cells
MMP	matrix metalloprotease
TGF	transforming growth factor
VEGF	vascular endothelial growth factor
PDGF	platelet derived growth factor
FGF	fibroblast growth factor
LOX	lysol oxidase
VEGFR1	vascular endothelia growth factor receptor 1
EREG	ereglin
COX-2	cyclooxygenase-2
TNC	tenascin C
EGF	epidermal growth factor
FNIII	fibronectin III repeats
ITH	intratumor heterogeneity
DKK2	dickkopf-2
CXCR4	chemokine receptor type 4
PI3K	PI3 kinase

LEF1	lymphoid enhancer binding factor 1
TCF	T-cell factor enhancer factor
RSPO	r-spondin
LGR5	leucine G-protein coupled receptor 5
BMP	bone morphogenic protein
TGFBR2	transforming growth factor beta receptor 2
TGFB1	transforming growth factor 1
COL1A1	collagen type 1 alpha 1 chain
IGF	insulin like growth factor
COL1A2	collagen type 1 alpha 2 chain
SPARC	secreted protein acidic and cysteine rich
PENK	pro-enkephalin
PCSK2/9	proprotein convertase subtilisin/kexins family
GSEA	gene set enrichment analysis
FN	fibronectin
PAPPA	pappalysin-1
GePS	Genomatix pathway system
SBE	smad binding element
ADAM	disintegrin and metalloprotease
CAM	chick chorioallantoic membrane
EBP	constitutively active beta-catenin
HUVEC	human umbilical vascular endothelial cells
bFGF	basic fibroblast growth factor

LSGS	low serum growth supplement
COG	children’s oncology group
FAK	focal adhesion kinase
LSD1	lysine-specific demethylase 1
PARP	poly ADP-ribose enzyme
PTHrP	parathyroid hormone related protein

Abstract

Metastasis remains the primary cause of death in cancer patients. The invasion-metastasis cascade is dependent on crosstalk between tumor cells and the tumor microenvironment, including stromal cells, extracellular matrix, and blood vessels, at both primary and distal sites. Ewing sarcoma is a bone and soft tissue tumor primarily affecting children and adolescents. Patients with only local disease have high survival rates of >70%. However, current treatment strategies for patients developing metastasis have dismal survival rates of <20%, making it imperative to better understand the biological processes that drive Ewing sarcoma metastasis. Wnt/beta-catenin activation is correlated with worse overall survival in Ewing sarcoma patients and tumor cell autonomous changes in cytoskeletal organization; however, the tumor cell non-autonomous changes induced by Wnt/beta-catenin remain unexplored. Extracellular matrix encoding genes and genes involved in tumor: tumor microenvironment crosstalk are among the genes activated by Wnt/beta-catenin signaling. Therefore, we hypothesized that Wnt/beta-catenin induces changes in the tumor microenvironment to promote Ewing sarcoma progression. The results of our studies have shown that activation of the Wnt/beta-catenin pathway led to increased secretion of structural collagens and matricellular proteins. Intriguingly, in the secretome of Wnt-activated Ewing sarcoma cells we also detected TGF-beta ligands and proteins that are downstream targets of the TGF-beta signaling cascade. We show that activation of Wnt

leads to derepression of TGFBR2, the key mediator of TGF-beta signaling, and detect discrete tumor cell sub-populations, responsive to both Wnt and TGF-beta ligands. Studies of Ewing sarcoma models, *in vitro* and *in vivo*, as well as in two independent patient cohorts, confirmed a direct relationship between beta-catenin signaling, TGF-beta activation, and angiogenesis in tumor cells. Mechanistically, this is due, in part, to increased endothelial cell proliferation mediated by increased secretion of the matricellular protein tenascin C. Additionally, we evaluated the other potential functions of the matricellular protein, tenascin C, in Ewing sarcoma progression, and identified stress-mediated activation of tenascin C through activation of Src kinase enhances an invasive phenotype. In conclusion, we have elucidated the importance of Wnt/beta-catenin signaling in altering tumor: tumor microenvironment interactions to promote Ewing sarcoma progression, through secretion of proteins such as tenascin C into the tumor microenvironment. These studies highlight tenascin C as a potential “achilles heel” and therapeutic target in Ewing sarcoma progression. Together these studies illustrate the critical contribution of tumor cell heterogeneity, cell plasticity, and tumor: tumor microenvironment crosstalk to sarcoma progression.

Chapter 1: Introduction

Thesis Overview

Ewing sarcoma is a bone and soft tissue tumor primarily found in children and adolescents. Currently there remains no effective treatment against metastatic Ewing sarcoma with 5-year survival rates for these patients being less than 20% (1). It is therefore imperative to understand the biological drivers of Ewing sarcoma metastasis to better identify therapeutic targets in hopes of improving survival for patients developing metastatic disease. Emerging research in the Ewing sarcoma field have implicated tumor: tumor microenvironment (TME) interactions to be key drivers of advanced disease (2). Additionally, Ewing sarcoma cells are plastic, and these cell state transitions are commonly driven by tumor heterogeneity and changes in the TME (3-6). In this thesis we dive deeply into the role of the Wnt/beta-catenin and TGF-beta pathways in dictating these tumor:TME interactions and provide support for both of these pathways in mediating transition to a more metastatic cell state.

Tumor metastasis

Tumor metastasis occurs when tumor cells disseminate from the local tumor and move to a distal site in the body to continue tumor growth (7). Metastasis is the primary cause of cancer related deaths in patients as there remains very few effective

treatments towards metastatic tumors (8). It is thought that metastasis occurs via the invasion-metastasis cascade beginning with invasion and intravasation from the primary tumor, dissemination into the circulation, survival in the circulation, extravasation into a distal site, formation of a micro metastatic site, and finally full outgrowth of the metastatic tumor. This process is accompanied by a variety of genetic and epigenetic alterations along with incorporation of non-neoplastic stromal cells. Additionally, the process is highly regulated by cell-cell interactions and cell-matrix interactions (7) (**Figure 1.1**).

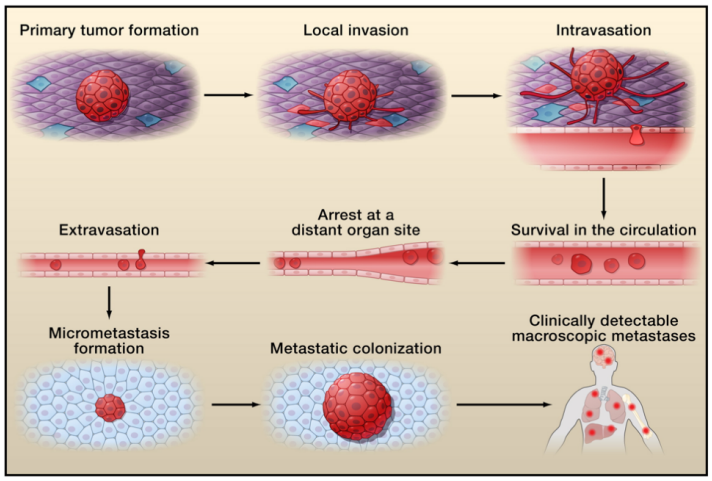

Figure 1.1 The invasion-metastasis cascade. Tumors undergo metastasis through the invasion-metastasis cascade depicted here. This figure is taken from Valastyan and Weinberg, 2011. Copyright permission received on November 6, 2018, license number 4463261434703.

A key driver of metastasis in carcinomas is the ability of cells to undergo the epithelial to mesenchymal transition (EMT) in which cells are able to phenotypically transition into a mesenchymal or more "metastatic" cell state via alterations in cell-cell interactions (9). Although debate continues as to whether or not EMT is necessary for metastasis, many studies have shown that EMT-like processes are able to contribute to changes in metastatic progression (10). EMT and metastasis are dictated by context specific mechanisms and the exact mechanism through which each cancer type metastasizes remains largely misunderstood. This introduction will specifically address the contribution of the TME and tumor heterogeneity to dictating metastatic progression

in solid tumors. Finally, we will discuss the specific contribution of tumor: TME crosstalk to dictating metastatic progression in Ewing sarcoma, a bone and soft tissue tumor with dismal survival for metastatic patients. Understanding these tumor:TME interactions will further advance our understanding of how Ewing sarcoma tumors are able to progress along the invasion-metastasis cascade, providing insight into development of new therapeutic targets to improve survival of patients with metastatic tumors.

The tumor microenvironment

The tumor is an ecosystem comprised of both tumor cells and the local TME made up of infiltrating non-neoplastic stromal cells, extracellular matrix (ECM), signaling ligands and cytokines, and blood vessels (11). Stromal cells is a very broad term that refers to all non-malignant cells found in a tumor, including but not limited to, fibroblasts, myofibroblasts, endothelial cells, adipocytes, mesenchymal stem cells (MSCs), macrophages, and many other immune cell types. Different stromal cells can secrete proteins, such as proteases or cytokines, that can degrade local ECM and/or signal to surrounding tumor and stromal cells to promote tumor growth (11). Additionally, tumor cells themselves are able to secrete proteins that can then signal to stromal cells to induce pro-tumorigenic changes. Most microenvironments in the body are not built to support tumor growth, however once the tumor is able to circumvent the normal microenvironmental cues, signaling from stromal cells and other components of the TME work together with tumor cells to drive tumor progression (12).Together this provides support for bidirectional control of tumor: TME interactions by tumor cells and

surrounding stromal cells to promote growth at the primary tumor and transition to a metastatic phenotype (**Figure 1.2**).

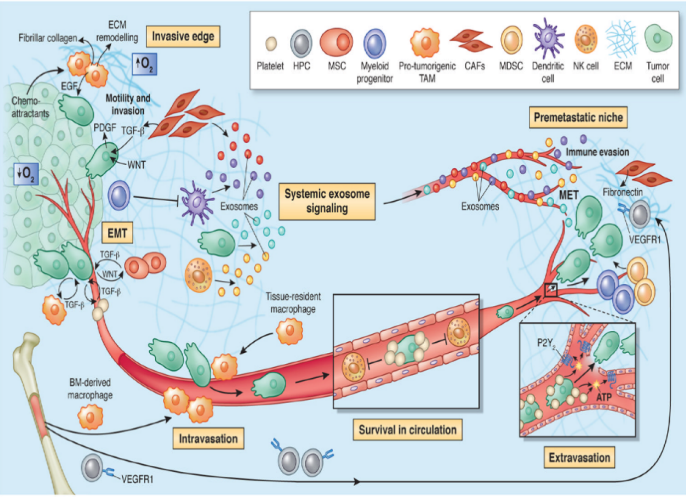

Figure 1.2 The tumor ecosystem. The tumor ecosystem is comprised of tumor cells and the local tumor microenvironment (TME). The TME contains blood vessels, infiltrating stromal cells, secreted extracellular matrix proteins, secreted ligands, and exosomes. Depicted here is the role of the TME in each stage of metastasis, where interactions at the local tumor lead to intravasation and invasion and secretion of systemic signals to the pre-metastatic niche. Blood vessels provide nutrients at the site of both primary and metastatic tumors and facilitate systemic dissemination in the circulation. This figure is taken from Quail and Joyce, 2013. Copyright permission received on October 17, 2018, license number 4451561219756.

The contribution of the tumor microenvironment to metastatic progression

In 1863, Rudolf Virchow identified infiltrating immune cells in cancer and linked inflammation to cancer (13). Since then, increased evidence of cancer in organs subject to chronic inflammation and the identification of infections found to be associated with 15% of human cancers (14, 15), solidified inflammation as a hallmark of cancer (16). This was the first report of the importance of the TME in mediating cancer progression. The majority of cancer research has focused on the cell autonomous changes that mediate tumor progression, but recently it has become clear that tumor cells are not the only factors dictating tumorigenesis.

Invasion and intravasation

Initial formation of a primary solid tumor begins with proliferation of only tumor cells, but as the tumor progresses and begins to grow, tumor cells come in contact with

stromal cells. This initial meeting of stromal cells to proliferating tumor cells results in a reactive stroma, increasing inflammation and mimicking a local environment found in wound healing. This reactive stroma results in secretion of signaling ligands into the TME that promote cell growth, thus creating a pro-tumor microenvironment (14).

The first step of metastasis requires cells to acquire an invasive phenotype and intravasate into a basement membrane (most carcinomas) or local ECM (sarcomas or mesenchymal derived tumors). Tumor cells or surrounding stromal cells secrete various proteases, such as matrix metalloproteases (MMP), that are able to degrade the ECM and liberate any growth factors sequestered in the local ECM (17). These growth factors can then signal back to the tumor cells and to other infiltrating non-malignant cells to activate an invasive signature. Typically changes in integrin signaling are observed, resulting in changes in cell-cell and cell-matrix adhesion leading to increased tumor cell motility (18).

There are multiple routes a tumor can take to increase invasiveness, all of which require cooperation between stromal cells, the ECM, and tumor cells. The first is most commonly observed in cancers of epithelial origin, such as carcinomas, and is known as collective invasion. This requires that cells move together as a unit through the local basement membrane. The second type of invasion is observed in mesenchymal cells and can occur in two different ways – protease, stress-fiber, and integrin-dependent invasion or protease, stress-fiber, and integrin independent Rho/Rock dependent amoeboid invasion. Tumor cells can easily transition between each of these invasive states and are not programmed to only undergo one form of invasion. The mechanism through which the tumor cell invades is dictated by the local TME. For example, many

carcinomas undergo EMT, thus can transition between the collective invasion to one of the mesenchymal single cell invasive mechanisms (19). The activation of EMT is mediated by various growth factors, most commonly the transforming growth factor beta ligand (TGF-beta) (to be discussed further in the TGF-beta signaling and Ewing sarcoma section), a ligand produced by the local TME (20). TGF-beta ligands can either be released from the ECM via protease degradation of the ECM or secreted by stromal cells, thus implying that changes in the local TME are necessary to promote EMT and subsequent invasion (21). This suggests a bidirectional relationship between the tumor cells and the TME that contribute to the invasive potential at a primary tumor site.

Dissemination and survival in circulation

A key limiting step to primary tumor growth, dissemination to the body, and growth at metastatic sites is access to blood supply (16). Tumor progression relies on the ability of cells to get nutrients from a blood source and therefore tumors can either induce neoangiogenesis, or formation of new blood vessels, a hallmark of cancer or co-opt existing vessels to gain access to nutrients (22, 23). Once cells have invaded the local ECM, they can enter these new blood vessels and disseminate into the circulation to reach distal sites in the body.

Formation of these blood vessels requires cooperation between the tumor cells and the local TME, in particular endothelial cells, pericytes, and bone marrow derived precursor cells (24). The process of neovascularization is a four-step process in which the initial area is wounded followed by local destruction and hypoxia, endothelial cells migrate after activation by angiogenic factors, proliferation of endothelial cells, and

finally, continual activation by angiogenic factors. These same steps occur in both tumor angiogenesis and physiological “normal” angiogenesis, however the resulting vessels are different (25). Tumor vessels are continuously undergoing reconfiguration as they constantly see angiogenic signals resulting in leaky blood vessels formed by weak interactions between adjacent endothelial cells (26). These leaky blood vessels are easily penetrated by tumor cells, allowing tumor cells to enter the blood stream and gain access to the rest of the body.

This process of angiogenesis in tumors is regulated by tumor cell and stromal cell secretion of pro and anti-angiogenic factors. A change in the balance towards increased pro-angiogenic factors, most prominent is the vascular endothelial growth factor (VEGF), and decreased anti-angiogenic factors, such as thrombospondins, is necessary to activate the angiogenic switch (25). Other pro-angiogenic factors critical in mediating angiogenesis include platelet derived growth factor (PDGF), fibroblast growth factors (FGF), and TGF-beta, among others (25). Much of the production of these factors comes from stromal cells, particularly tumor associated macrophages, mesenchymal stem cells in the area, and cancer associated fibroblasts (11). This alters the balance of the local TME to be pro-angiogenic, resulting in activation of endothelial cells, leading to endothelial cell production of MMPs to break down the ECM and induce migration of endothelial cells (25). This process is known as the angiogenic switch (22). This angiogenic switch is primarily mediated by expression and secretion of members of the AngioMatrix by both tumor and stromal cells (27), implying a critical role for tumor: TME signaling in promoting blood vessel formation and subsequent metastatic progression.

Once in the circulation, tumor cells must survive in circulation and then extravasate into a distal site. Only 0.01% of cells that enter circulation are able to form a metastatic colony at a distal site (28). The first step in making it to the distal site is survival in circulation, also dependent on stromal cells and interactions with the microenvironment (29). Platelets found in blood vessels are able to aggregate to tumor cells circulating in blood vessels and protect tumor cells from natural killer cell mediated cytotoxicity and immune evasion. This aggregate of platelets and tumor cells can then circulate through the blood system until arrival and docking at a distal site (30). Ability to enter and survive in the blood stream is therefore dependent on tumor cells, the local TME, and non-stromal cells circulating in blood vessels.

Extravasation

Once at the distal site, tumor cells must be able to extravasate into this new site to allow for metastatic colonization. Platelets present in tumor cell aggregates interact with the local endothelium through integrin attachment to the extracellular matrix at the distal site of metastatic outgrowth. Platelets specifically open the endothelial barrier via ATP-dependent activation of endothelial cell surface receptors, allowing for metastatic seeding (31).

Additionally, tumor cells can send signals to distal sites, leading to formation of a pre-metastatic niche favorable to extravasation of tumor cells. This occurs through secretion of systemic signals from the tumor cells themselves such as an increase in lysol oxidase (LOX) secretion that activates organ specific upregulation of fibronectin (32). This induces mobilization of vascular endothelial growth factor receptor positive

(VEGFR1+) bone marrow derived progenitor cells. The organ specific upregulation of fibronectin serves as a docking site for VEGFR1+ cells leading to alterations in local signaling to create a pre-metastatic niche prior to tumor cell arrival (33, 34). Thus, the cooperation between stromal cell and tumor cell signaling and interactions with the local microenvironment at the distal site mediates extravasation and the ability to form a metastatic site.

Metastatic outgrowth at a distal site

Although metastasis accounts for many tumor related deaths, the process of metastasis is highly inefficient (29). The rate limiting step of the invasion-metastasis cascade is the ability to establish a micro metastatic colony at a distal organ site (35). Once tumor cells leave a primary tumor site they have left a tumor promoting local microenvironment but encounter a microenvironment not supportive of tumor growth. They must be able to survive in this foreign microenvironment and learn to adapt the TME to be conducive to metastatic outgrowth.

These micro metastatic colonies must gain access to the local blood supply to support growth by activating the angiogenic switch at the distal site. Once at a distant site, the tumor cells interact with a physiological blood vessel that is not leaky and must permeabilize the vessel (36). This occurs through secretion of proteins such as ereglin (EREG), cyclooxygenase-2 (COX-2), various MMPs, and VEGF, which can disrupt the the local vasculature and increase permeability for tumor cells to gain access to nutrients (36). Once the vessel walls have been permeabilized, the tumor is able to alter signaling in the new microenvironment to lead to sprouting of neovessels and

production of periostin and TGF-beta to continue vascular growth and support tumor growth (37). Based on this evidence, it is clear that the invasion-metastasis cascade and tumor progression relies on altered tumor: TME crosstalk at both the primary and distal tumor site.

The role of extracellular matrix components in creating a tumor "niche"

In 1889, Stephen Paget proposed the "seed and soil" hypothesis, stating that tumor cells were able to disseminate to all places in the body, but they needed the right local "soil" to stick and form a metastatic outgrowth (38). This means that cells need to be able to find the right foreign microenvironment and that the ability to form micro metastasis is dictated by the ability to convert the new microenvironment to a metastatic niche. The necessary changes in the local microenvironment and the ability of the cells to interact with this distal environment, is dependent on the type of cancer (33). This is evident through variable propensities of different cancer types to disseminate to particular locations (38). For example, in Ewing sarcoma, metastasis is most commonly found in the lung, bone, or bone marrow (1), while breast cancer commonly metastasizes to the brain, lymph nodes, lung, and liver (39). This concept is known as metastatic tropism and is dictated by the ability to form a pre-metastatic niche supportive of that particular cancer cell growth.

Creation of a supportive niche is mediated by composition and structure of both the local and metastatic ECM (11). At the site of primary tumor formation, deposition and organization of ECM components, such as various types of collagen, can dictate prognosis. For example, increased matrix stiffness and collagen fibril formation in breast

cancer results in poor clinical outcome (40). Additionally, high expression of protease inhibitors to block degradation of the ECM can be a biomarker for good prognosis of solid tumors (40). The tumor can also hijack the local TME and alter ECM synthesis and degradation to be conducive of tumor growth. One example of this is in osteosarcoma where the tumor resides in the bone, a microenvironment dominated by collagen I. Osteosarcoma tumors will alter the balance of collagen synthesis and degradation by osteoblasts and osteoclasts respectively, leading to a change in the local ECM structure to be pro-tumorigenic (41). This shift in the collagen production balance also results in changes in growth factor availability as they are sequestered in the collagen matrix and can be released during collagen degradation (33). In bone tumors, such as Ewing sarcoma, where there is an increase in the osteolytic activity, or bone matrix degradation, the result is an increase in bioavailability of growth factors, altering the signaling to the local tumor cells as well as non-malignant cells in the TME.

The ECM can also play a role at distal sites in the metastatic niche. Here, the group of ECM proteins known as the matrisome, are critical in dictating progression from a micro metastasis into a metastatic outgrowth (42). The composition of the matrisome is comprised of secreted proteins from both the tumor cells as well as the stroma. One of the limiting aspects of metastatic growth at a distant site is access to a blood supply (16). Therefore, metastatic sites need to establish a blood source through neoangiogenesis, or vessel formation, by activating the angiogenic switch (22). A subset of matrisome proteins, categorized as the angiomatrix, have been shown to be critical in dictating the angioswitch (27). This provides one example of where the ECM at the distal site can be critical in controlling metastatic colonization and growth.

Tenascin C as a mediator of tumor: TME interactions in cancer

Tenascin C (TNC) is a matricellular protein found in the ECM that is a mediator of cell and ECM interactions. *TNC* is highly expressed during development in motile tissues, sites of EMT and branching morphogenesis, and dense connective tissue such as tendons and ligaments. However, it is only expressed in adults in instances of wound repair or in stem cell niches (43). TNC contains at least one epidermal growth factor (EGF) binding domain followed by a string of 9-16 fibronectin III repeats (FNIII). Alternative splicing occurring in 9 of these FNIII domains gives rise to a potential of 511 different isoforms. Each of these FNIII domains have specific binding partners, proteolytic cleavage sites, and post-translational modification sites, making the functions of TNC very diverse (44).

TNC is categorized as a matricellular protein because it can both bind to ECM proteins, most predominantly fibronectin, as well as cell surface receptors, such as integrins. It can also interact directly with ligands in the microenvironment, such as Wnt3a and TGF-beta. TNC contributes to the composition of the local ECM and defines stiffness of the local environment. It also influences cellular processes including, but not limited to adhesion, cell spreading, invasion, and migration (43). Activation of *TNC* expression has been shown to be regulated by a variety of pathways, ligands, and transcription factors including through the TGF-beta pathway, Notch pathway, PDGF signaling, and transcription factors ETS1 and ETS2. *TNC* can also be mediated through mechanical stresses in the microenvironment and changes in matrix stiffness (45).

TNC has been shown to be expressed by both tumor cells and surrounding stromal cells in the TME. High expression of *TNC* has been observed in a variety of solid tumors and has been predominantly found at the invasive front of tumors (46). TNC is an important contributor to formation of the pre-metastatic niche in breast cancer (47). TNC is a poor prognosis indicator in colorectal cancer (48), and *TNC* is necessary for lung engraftment in Ewing sarcoma (3). These studies and others implicate that TNC is important for metastatic progression of solid tumors and provide further evidence for ECM proteins as drivers of metastasis.

Tumor heterogeneity, cell plasticity, and the tumor microenvironment

Genetic tumor heterogeneity

One of the major drivers of these changes in the tumor cells and their interactions with the tumor microenvironment, is intratumor heterogeneity (ITH). ITH can be driven by genetic or epigenetic heterogeneity in tumor cells. Genetic heterogeneity occurs when subpopulations of cells within a tumor contain different DNA mutations and copy number alterations, resulting in varying mutational burden. Genetic ITH was first described in 1958 by Julian Huxley but was not fully defined until the invention of next generation sequencing that allowed for massive DNA sequencing of patient tumor biopsies. Use of next generation sequencing on tumors allowed for identification of tumors comprised of multiple subpopulations with different mutations (49).

Detection of genetic ITH through sequencing can also be used to define temporal order of events and reconstruct phylogenetic trees that show the evolution of each genetically similar subpopulation in the tumors (50). There are multiple theories on how

ITH arises, including the concept of classic Darwinian evolution within a tumor, where all cells are derived from one original cell (51), or through parallel evolution of different original subclones (52). Additionally, researchers have studied the proportions of different heterogeneous subpopulations to posit that these genetically diverse subpopulations are formed through a balance between survival of the fittest clone and neutral outgrowth of clones (49). Recent research has also hypothesized the existence of clonal cooperativity, where subclones work together to promote phenotypes necessary for survival and growth of the tumor (53).

Epigenetic tumor heterogeneity

A second mechanism through which ITH occurs, is epigenetic ITH (54). Epigenetics is defined as a stable and heritable change in gene expression occurring without any changes in DNA sequence (55). A key aspect of the epigenome is how nucleosomes are positioned in relationship to the DNA, thus controlling transcription of the DNA via changes in DNA methylation, chromatin remodeling, and histone modifications (56). Each of these processes can result in changes in gene expression, leading to changes in phenotypic cell states. For example, over-activity of the polycomb group proteins leads to increased gene silencing of polycomb targets in many cancers (57). A key aspect of epigenetic ITH is its reversibility, meaning it is constantly in a changing state, making it hard to detect when taking only a snapshot of a tumor through a biopsy (54).

Tumor heterogeneity and the tumor microenvironment

A major contributing factor to both genetic and epigenetic tumor heterogeneity, is the TME. The ability of fit clones to survive in the tumor is dependent on the TME and changes in the TME have been shown to drive genetic heterogeneity (58). A taxonomy report from several different cancer cell types showed that cell and tissue of origin can influence ITH and mutational burden, suggesting that the TME is a driver of ITH (59). Additionally, changes in the local TME can signal to induce changes in cell states resulting in epigenetic ITH. In particular, deregulation of the local ECM has been shown to induce ITH (60). Changes in the tumor cells are constantly occurring throughout the invasion-metastasis cascade, both at the genetic and epigenetic level, as the cells are undergoing multiple rounds of genetic diversification followed by clonal selection until a metastatic clone is developed (7).

Cell plasticity and the TME

Cell plasticity refers to the ability of cells to transition between different cell states. This implies that the cell state is reversible, and is mainly dictated by changes in cell phenotype and gene expression. The most predominant example of cell plasticity is the EMT pathway. EMT switching is a very plastic and reversible program as cells can lie anywhere on the spectrum between epithelial and mesenchymal. Activation of this program, as well as other programs that dictate phenotypic plasticity, is mediated by changes in cell signaling pathways that alter expression of driver transcription factors and mediate global transcriptomic changes (9). In particular, for EMT, activation of the TGF-beta, Wnt, and NOTCH pathways all increase expression of key transcription

factors, *SLUG, SNAIL, TWIST, ZEB1*, and *ZEB2.* These transcription factors can then in turn alter transcription of key genes necessary for the EMT, resulting in upregulation of cell-matrix junctions and downregulation of genes necessary for cell-cell junctions, such as E-cadherin (9, 20). Varying combinations of EMT transcription factor activation can control where on the EMT spectrum the cell lies, and changes in activation of these factors can then result in reversion through the mesenchymal epithelial transition.

Signals present in the TME, coming from both tumor cells and the local TME, activate changes in gene expression that lead to reversible phenotypic changes, such as EMT, increasing the invasive phenotype. This is a prime example of the tumor and the TME work together to alter cell state and induce a metastatic cell state. However, EMT is not the only case in which this is occurring. Secretion of proteins from surrounding stromal cells can induce phenotypic changes, such as increased motility of tumor cells, independently of the EMT pathway (11). For example, tumor associated macrophages regulate the production of fibrillar collagen which accelerates tumor motility in breast cancer (61). Another example is in Ewing sarcoma, where microenvironmental stresses, such as serum starvation and hypoxia, can induce changes in CXCR4 levels, a key chemokine receptor whose increased expression results in increased migration and invasion (4).

Tumor heterogeneity, cell plasticity, and the tumor microenvironment are all intimately linked and work together to control progression along the invasion-metastasis cascade. A prime example of all three of these concepts merging to drive invasion was identified in 2013 in breast cancer. Here, Chaffer et al., showed that *ZEB1*, a crucial EMT transcription factor, is held in a bivalent promoter state in basal-type epithelial

breast cancer cells, but not in luminal breast cancer cells. Bivalency is present when the promoter has both the activating H3K4me3 and repressive H3K27me3 mark, meaning it is poised for rapid activation of the *ZEB1* promoter. However, in the luminal cells, the promoter only displays the H3K27me3 repressive state, meaning basal cells, but not luminal cells, can activate the EMT phenotype in the presence of TGF-beta because *ZEB1* is poised for activation (62). This is a key example in which epigenetic plasticity can drive response to the TME and thus mediate phenotypic plasticity. The literature discussed to this point show that the ability of a tumor to initiate and progress along the invasion-metastasis cascade is clearly dependent on tumor heterogeneity, cell plasticity, and the TME all working together (**Figure 1.3**).

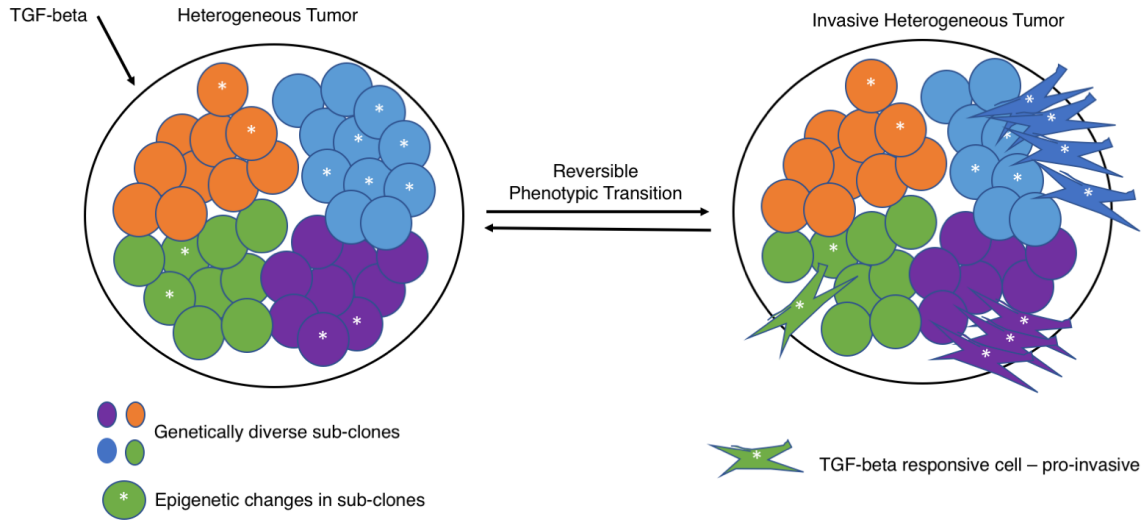

Figure 1.3 Tumor heterogeneity and cell plasticity in metastasis. Both genetic and epigenetic heterogeneity contributes to tumor heterogeneity. Subpopulations of genetically and epigenetically diverse clones exist in the tumor. When exposure to microenvironmental stimuli, such as TGF-beta ligands, occurs, cells can undergo phenotypic transitions. The ability of cells to respond to stimuli is dictated by genetic and epigenetic state. Phenotypic transitions are reversible, known as cell plasticity, and can lead to pro-invasive cell states, such as in the case of the epithelial to mesenchymal transition.

Ewing sarcoma

Ewing sarcoma is the second most common bone and soft tissue tumor that predominantly affects children and adolescents. It is characterized by a small round

blue cell histology and is of presumed stem cell origin, however the exact cell of origin remains to be determined. There are only 200 new cases in the United States per year, with a predilection for males and a peak incidence between the ages of 5 and 25. Primary tumors tend to form in the long bones (femur, humerus, and tibia), pelvis, and chest wall, while metastasis prefers the lungs, bone, and bone marrow (1, 63) (**Figure 1.4A-B**).

Ewing sarcomas are characterized by the pathognomic fusion of EWS, an RNA binding protein, with an ETS family member, a family of transcription factors, with the most common fusion being EWS-FLI1 in 85% of patients (64). The chromosomal translocation creating this fusion occurs between chromosome 11 (FLI1) and chromosome 22 (EWS) t(11;22). Other fusions that have been reported include EWS-ERG, EWS-ETV1, and EWS-E1AF (1). This results in the binding of the DNA binding domain of the ETS family members with EWS, an RNA binding protein whose RNA binding domain is lost in the fusion. Other than this characteristic fusion gene and occasional STAG2, p53, and CDKN2A mutations, there are no other recurrent mutations in the Ewing sarcoma genome and the genome is relatively silent in comparison to other tumors (65-67). EWS-FLI1 is the driving oncogene and acts as a pioneer factor, resulting in global reprogramming of the epigenome through creation of de novo enhancers at GGAA microsatellites present in the genome. This global reprogramming leads to changes in gene expression, transforming the cell into an oncogenic cell state (68, 69) (**Figure 1.4C**).

Patients that only develop a primary tumor have a high survival rate of > 70%, however patients that develop metastasis have a very low survival rate of < 20% (63)

(**Figure 1.4D**). Treatment currently includes a combination of aggressive chemotherapy and local control of radiation and/or surgery. Chemotherapy consists of many rounds of vincristine, doxorubicin, cyclophosphamide, ifosfamide, and etoposide (1). However, this is not enough for the third of patients who develop metastasis, making it imperative to identify the biologic drivers of Ewing sarcoma metastasis to better treat those patients who develop metastasis.

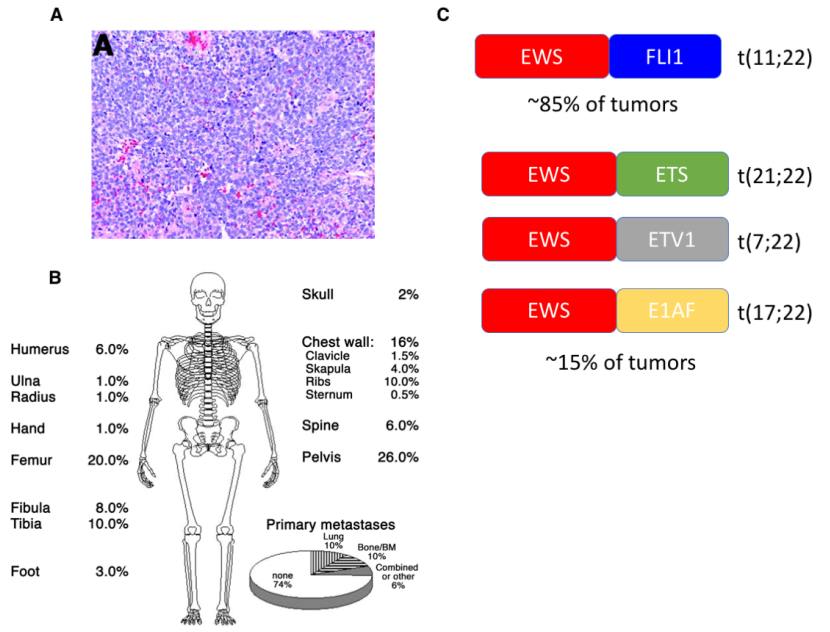

Figure 1.4 Ewing sarcoma. A. Ewing sarcoma is characterized by small round blue cell histology. **B.** Anatomical distribution of Ewing sarcoma tumors at diagnosis and at presence of metastasis. **C.** Ewing sarcoma tumors all contain the pathognomic EWS/ETS fusion. EWS-FLI1 is found in 85% of tumors but other binding partners include ETS, ETV1, and E1AF. A. and B. are taken from Bernstein et al., Ewing's sarcoma family of tumors: current management, *The Oncologist,* 2006. Copyright permission received on November 10,2018.

Ewing sarcoma metastasis

Although much of Ewing sarcoma research has focused on understanding the role of EWS-FLI1 in driving tumorigenesis, very little is known about the drivers of Ewing sarcoma metastasis. As stated previously, the Ewing sarcoma genome is genetically silent and very few recurrent mutations have been identified, regardless of prognosis. This makes it very probably that the drivers of metastasis will not be identified through next generation sequencing efforts, and focus should be on understanding the biological processes that could be driving the metastatic phenotype.

Few studies have identified driver pathways of Ewing sarcoma metastasis, and no therapeutic targets have been successfully exploited through identification of these few pathways. CXCR4 has been shown to promote invasion and migration (4). Dickkopf-2 (DKK2) was shown to promote metastasis *in vivo* through upregulation of the pro-metastatic genes *CXCR4, PTHrP, RUNX2, TGFB1,* and *MMP1 (70)*. ERBB4, a key receptor that mediates EGF signaling, was identified to be upregulated through increased activation of the PI3 kinase (PI3K)-Akt pathway resulting in enhanced chemoresistance, invasion, and migration (71). Additionally, *ERBB4* had increased expression in cell lines derived from recurrent or metastatic tumors in comparison to their matched primary tumor (72). More recently, *EWS-FLI1* was identified to be expressed at varying levels in individual cells within a cell line. Cells categorized as *EWS-FLI1* low expressing cells are more invasive and metastatic than *EWS-FLI1* high cells, suggesting the possibility that EWS-FLI1 itself could be contributing to the metastatic phenotype (73). Finally, identification of the Wnt/beta-catenin pathway activation to be correlated with lower overall survival and lower event free survival implicates the Wnt/beta-catenin pathway as a metastatic driver, to be discussed further (3).

Ewing sarcoma and the tumor microenvironment

Crosstalk between Ewing sarcoma tumor cells and the local TME has been poorly studied. Sarcomas are derived from mesenchymal cells and do not directly sit on top of a basement membrane, while carcinomas are derived from epithelial cells and grow on top of a basement membrane. Carcinomas must then invade the local

basement membrane, while sarcomas grow within the ECM and soft tissue. Therefore, much of the local TME is dictated by ECM or infiltrating cells. Multiple studies have implicated the Ewing sarcoma TME to be important in dictating metastatic progression. Microarray profiling of 46 tumors identified a poor prognosis signature comprised of 33 genes, however this prognosis signature was only identifiable when tumors with high stromal content were present in the analysis (2). In support of this, subcutaneous xenografts of Ewing sarcoma have been shown to develop spontaneous metastasis at a much lower rate than orthotopic xenograft models, suggesting that the bone TME is much more supportive of metastatic progression (74).

Inside the bone TME, the “vicious” cycle works to maintain a pro-tumor environment by altering the balance between bone formation, mediated by osteoblast activation, and bone resorption, mediated by osteoclasts (75). Ewing sarcoma tumors in the bone are characterized by extensive bony remodeling mediated by an osteolytic phenotype (70). In an attempt to inhibit the osteolytic activity in the TME, groups have utilized bisphosphonates to inhibit osteoclast activity in preclinical models of Ewing sarcoma and show decreased tumor development in bone sites and increased overall survival in mice (76). Specific pathways in the bone TME have also been shown to drive Ewing cell motility and invasion, such as signals from fibroblast growth factor (FGF) via the FGFR1-PI3K-Rac1 pathway, implicating the bone TME as a driver of metastasis (77).

In addition to the bone TME, changes in microenvironmental stresses and induction of angiogenesis have been shown to promote Ewing sarcoma progression. The combination of serum starvation and hypoxia induces migration and invasion via

the CXCR4 signaling axis (4). These same microenvironmental cues lead to activation of Src kinase and subsequent formation of actin rich protrusions, or invadopodia structures (6). Hypoxia alone can also alter EWS-FLI1 levels to promote the metastatic phenotype (78).

Another response to lack of nutrients and hypoxic conditions in cancer is the induction of angiogenesis. In Ewing sarcoma specifically, bone marrow cells are able to migrate to the tumor and differentiate into endothelial cells and pericytes to promote angiogenesis in the bone TME (79, 80). This is partially mediated by the CXCR4 signaling axis, as blocking CXCR4 signaling reduces PDGF-B protein expression and decreases efficiency of vascular formation in the bone TME (81). Further understanding about how both changes in the TME as well as changes in the tumor cells themselves could help identify critical interactions between tumor cells and the TME in driving Ewing sarcoma progression.

Wnt/beta-catenin signaling and Ewing sarcoma

The Wnt/beta-catenin signaling axis is a highly conserved pathway important in developmental processes such as cell proliferation, morphological changes, and cell organization. There are 19 different Wnt ligands, that can activate both canonical Wnt signaling occurring through beta-catenin activation, or non-canonical signaling which are beta-catenin independent signaling pathways. Due to findings that activation of the Wnt/beta-catenin pathway is a biomarker for aggressive disease in Ewing sarcoma, we will only focus on the canonical Wnt/beta-catenin pathway throughout the remainder of this thesis. Binding of Wnt ligand to a frizzled receptor results in recruitment of the

destruction complex (Axin2, Disheveled, APC, and GSK3-beta), freeing up beta-catenin to accumulate in the cytoplasm and translocate into the nucleus. Once beta-catenin is in the nucleus, it binds to various co-factors, most predominantly TCF and LEF, that facilitate binding to DNA and global changes in transcription. This signaling can be further potentiated by addition of secreted R-spondins (RSPO) which binds to the coreceptors, leucine G-protein coupled receptors, LGR4 and LGR5, to enhance Wnt signaling (82).

These Wnt-mediated global changes in transcription are context and cell-dependent. In Ewing sarcoma, activation of the Wnt/beta-catenin pathway induces robust expression of the lymphoid enhancing factor 1 (*LEF1*), *AXIN2*, and *NKD2*, among others, while classic Wnt targets, *MYC* and *CCND1*, remain unaltered (3). Previous to beginning work discussed in this thesis, our lab had identified the stem cell marker *LGR5* to be expressed by Ewing sarcoma cells and high *LGR5* expressing Ewing sarcoma cells to be Wnt responsive (83). Furthermore, activation of the Wnt pathway through addition of exogenous Wnt ligands, in particular Wnt3a, leads to heterogeneous activation of the pathway in cell lines. Activation of this pathway prior to injection into mouse via tail vein resulted in increased lung engraftment and high expression of *LEF1* in patients is correlated with worse event free and overall survival (3). More recently, inhibition of Wnt signaling through inhibition of porcupine, a mediator of Wnt ligand secretion, was shown to increase survival in preclinical mouse models (84). Our lab has also shown that activation of the Wnt/beta-catenin pathway antagonizes EWS-FLI1, resulting in derepression of many EWS-FLI1 targets (3). These studies implicated that Wnt/beta-catenin alters tumor cell-autonomous functions to

increase metastatic potential, through in vivo studies showing Wnt/beta-catenin activated cells had increased time to tumor engraftment. However, it remains to be determined if and how activation of Wnt/beta-catenin alters tumor cell non-autonomous functions (**Figure 1.5**).

Transforming growth factor beta and Ewing sarcoma

The transforming growth factor beta (TGF-beta) pathway regulates development through processes such as cell growth, adhesion, migration, apoptosis, differentiation, and angiogenesis. The TGF-beta superfamily is comprised of 30 members that include TGF-beta ligands, most commonly TGFB1, TGFB2, and TGFB3, bone morphogenic proteins (BMPs), growth and differentiation factors, activins, and nodals. Here, we will focus on the canonical TGF-beta pathway in which TGF-beta ligands, such as TGFB1, bind to a type 2 receptor (TGFBR2) leading to dimerization with a type 1 receptor (ALK4, ALK5, or ALK7). Formation of the TGFBR2/type1 receptor complex leads to

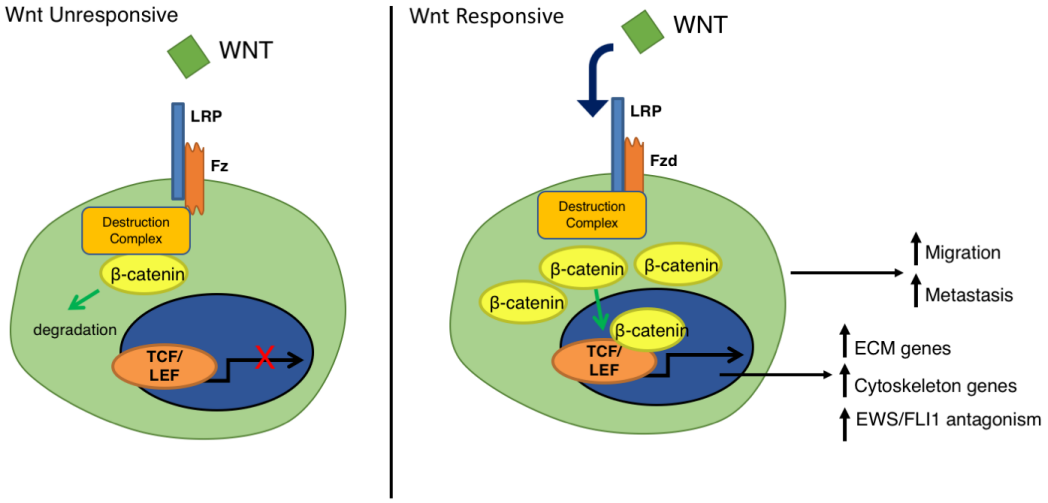

Figure 1.5 Wnt/beta-catenin signaling in Ewing sarcoma. Ewing sarcoma cells that are unresponsive to Wnt ligands are unable to induce beta-catenin accumulation in the nucleus and therefore do not activate Wnt target genes. Ewing sarcoma cells that are responsive to Wnt ligands lead to activation of Wnt targets, including extracellular matrix and cytoskeleton genes. Wnt activation results in EWS/FLI1 antagonism and increased migration and metastasis.

phosphorylation of downstream activating smads, SMAD2 or SMAD3. These phosphorylated smads dimerize with SMAD4 before translocation into the nucleus where they can directly bind to specific DNA sequences in promoters, resulting in global transcriptomic changes. Three different ligands for the TGF-beta pathway, TGFB1, TGFB2, and TGFB3 can all signal through the TGFBR2/type1 receptor complex, while other ligands such as bone morphogenic proteins (BMP), activins, and nodals, activate one of the other 5 type 2 receptors (21). We are interested specifically in the role of TGFB1/2/3 for the remainder of this thesis and will only discuss this pathway in the context of the canonical smad pathway activated through the TGFBR2/type1 receptor complex.

The TGF-beta pathway remains a poorly studied pathway in Ewing sarcoma. Initial studies identified *TGFBR2* to be repressed by EWS-FLI1, rendering Ewing sarcoma cells unable to activate the downstream smad cascade in response to TGF-beta ligands. Simultaneously, it was shown that overexpression of this receptor in Ewing sarcoma blocked tumor growth in a subcutaneous model, suggesting that expression of *TGFBR2* would be tumor suppressive in Ewing sarcoma (85). Since this finding, the paradoxical role of TGF-beta as a tumor suppressor and tumor promoter has been identified in carcinomas. At earlier stages of tumor formation, activation of TGF-beta is associated with apoptosis, resulting in a block of tumor growth (86). However, TGF-beta is a critical driver of the EMT phenotype, leading to increased invasion and cell motility as the tumors progress (20). Although the traditional EMT pathway does not occur in Ewing sarcoma, sarcoma cells have been shown to be able to exist in a metastable state where they can transition between epithelial-like and mesenchymal-like

phenotypes (87). This implies that perhaps scenarios in which *TGFBR2* is derepressed at later stages of Ewing sarcoma tumor progression could then activate this mesenchymal-like or more metastatic phenotype. It is therefore imperative to understand the role of TGF-beta at all stages of tumor progression in Ewing sarcoma, as it could be a critical driver of metastasis, post primary tumor formation (**Figure 1.4**).

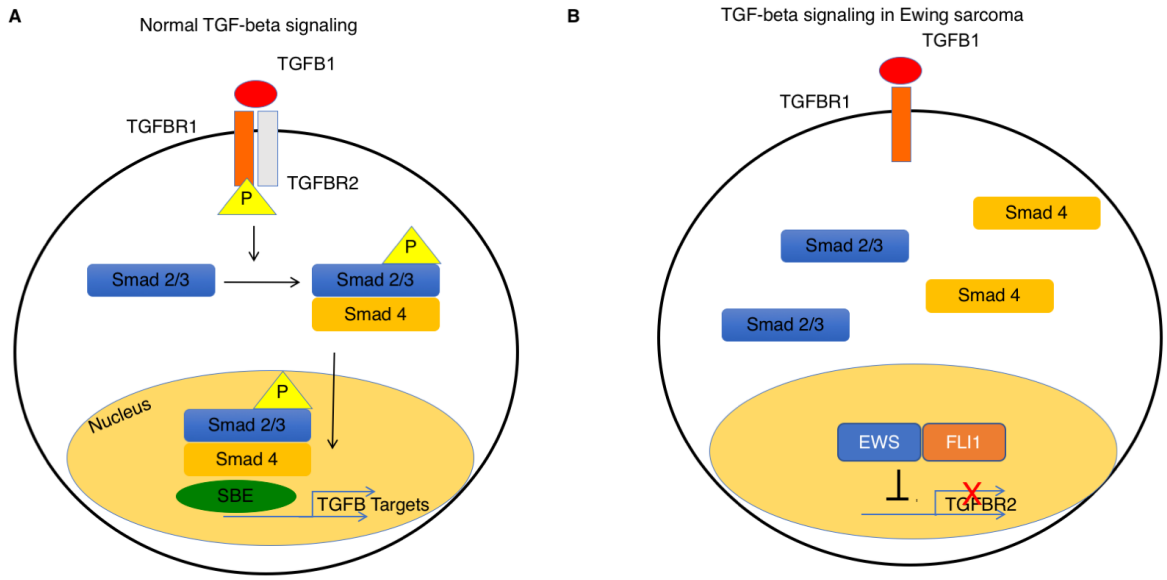

Figure 1.6 TGF-beta signaling in Ewing sarcoma. A. In TGF-beta responsive cells, i.e. not Ewing sarcoma cells, TGFB1 ligands bind to the TGF-beta receptors activating SMAD phosphorylation and ultimately transcription of TGF-beta targets. B. In Ewing sarcoma, EWS/FLI1 represses TGFBR2, leaving Ewing sarcoma cells unable to respond to TGF-beta ligands and activate any TGF-beta target genes.

Linking Wnt, TGF-beta, and the tumor microenvironment in Ewing sarcoma

As discussed here, two potential factors contributing to Ewing sarcoma metastasis are the Wnt pathway and contributions from the local TME. However, unlike many colorectal tumors where activation of Wnt signaling is dictated by a recurrent mutation leading to constitutive activation of the pathway, there are no recurrent mutations in the Wnt/beta-catenin pathway in Ewing sarcoma (65-67, 88). Additionally, we did not detect presence of any secreted Wnt ligands in unmodified Ewing sarcoma cells (89). This implies that activation of this pathway is most likely occurring when

tumor cells are coming into contact with Wnt ligands produced by stromal cells in the TME.

Additionally, we show in this thesis that activation of the Wnt/beta-catenin pathway is associated with increased expression of genes that encode structural components of the ECM, suggesting a link between activation of the Wnt pathway and changes in the TME (3). The remaining question is then what are these microenvironmental changes and do they play a role in mediating the metastatic phenotype associated with activated Wnt signaling? Lastly, activation of the Wnt/beta-catenin pathway leads to antagonism of EWS-FLI1 and derepression of many repressed EWS-FLI1 targets, including *TGFBR2 (3)*. This begs the question of whether or not activation of Wnt is able to prime these cells to be TGF-beta responsive and if so, does this also play a role in the Wnt activated phenotype? Answering all of these questions will elucidate the connection between Wnt, TGF-beta, and the TME in promoting Ewing sarcoma progression in hopes of identifying novel therapeutic targets and ways to exploit tumor: TME crosstalk to improve overall patient survival.

Summary and Aims

Ewing sarcoma patients that develop metastatic disease have dismal survival rates, making it imperative to better understand the biological drivers of metastasis in hopes of identifying new therapeutic targets (1). Activation of the Wnt/beta-catenin pathway is associated with transition to a more metastatic cell state and global transcriptomic changes, specifically increased expression of ECM associated genes (3). Therefore, we hypothesized that activation of the Wnt/beta-catenin pathway is altering

the tumor: TME crosstalk to promote a metastatic phenotype. In this thesis we address this hypothesis in the following three aims.

<u>Aim 1:</u> Identify the Wnt mediated contribution of Ewing sarcoma cells to the local TME through identification of the Ewing sarcoma secretome.

<u>Aim 2:</u> Determine the link between the Wnt and TGF-beta pathways in driving metastasis, specifically through secretion of proteins important for angiogenesis.

<u>Aim 3:</u> Investigate the role of the secreted protein, Tenascin C, in driving changes in tumor: TME crosstalk through Src-mediated invasion.

Understanding how the Wnt/beta-catenin and TGF-beta pathways dictate changes in tumor: TME interactions will help elucidate how to best target the TME in hopes of both preventing metastatic disease progression and improving survival of patients who have developed metastatic disease.

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Chapter 2: The Ewing sarcoma secretome and its response to activation of Wnt/beta-catenin signaling*[1]

Summary

Tumor: tumor microenvironment (TME) interactions are critical for tumor progression and the composition and structure of the local extracellular matrix (ECM) are key determinants of tumor metastasis. We recently reported that activation of Wnt/beta-catenin signaling in Ewing sarcoma cells induces widespread transcriptional changes that are associated with acquisition of a metastatic tumor phenotype. Significantly, ECM protein-encoding genes were found to be enriched among Wnt/beta-catenin induced transcripts, leading us to hypothesize that activation of canonical Wnt signaling might induce changes in the Ewing sarcoma secretome. To address this hypothesis, conditioned media from Ewing sarcoma cell lines cultured in the presence or absence of Wnt3a was collected for proteomic analysis. Label-free mass spectrometry was used to identify and quantify differentially secreted proteins. We then used in silico databases to identify only proteins annotated as secreted. Comparison of the secretomes of two Ewing sarcoma cell lines revealed numerous shared proteins, as well as a degree of heterogeneity, in both basal and Wnt-stimulated conditions. Gene

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set enrichment analysis of secreted proteins revealed that Wnt stimulation reproducibly resulted in increased secretion of proteins involved in ECM organization, ECM receptor interactions, and collagen formation. In particular, Wnt-stimulated Ewing sarcoma cells upregulated secretion of structural collagens, as well as matricellular proteins, such as the metastasis-associated protein, tenascin C (TNC). Interrogation of published databases confirmed reproducible correlations between Wnt/beta-catenin activation and *TNC* and *COL1A1* expression in patient tumors. In summary, this first study of the Ewing sarcoma secretome reveals that Wnt/beta-catenin activated tumor cells upregulate secretion of ECM proteins. Such Wnt/beta-catenin mediated changes are likely to impact on tumor: TME interactions that contribute to metastatic progression.

Introduction

The local and metastatic progression of solid tumors is critically dependent on interactions and crosstalk between tumor cells and their local tumor microenvironment (TME). Both cellular and non-cellular components of the TME can bind and activate surface receptors on tumor cells to impact on cell signaling and cell behavior (1). In particular, the proteinaceous extracellular matrix (ECM) is a key player in tumor: TME crosstalk and changes in the composition and structure of the ECM can profoundly alter cell signaling (2). The main source of ECM proteins in the TME is secretion from fibroblasts (3). However, secretion of ECM proteins also occurs in normal physiological processes, such as secretion of collagens from osteoblasts during bone formation (4). In cancer, aberrant secretion of proteins from non-tumor stromal cells, as well as tumor cells themselves, can disturb homeostatic signaling and promote disease progression.

Indeed, each of the major hallmarks of cancer (5) is impacted by secreted proteins in the TME - i.e. VEGF and it's role in angiogenesis, MMPs and their role in matrix degradation, and cytokines and their recruitment of immune cells (6).

Ewing sarcoma is an aggressive tumor of bone and soft tissue that has a peak incidence in adolescents and young adults (7). Although much has been learned about the genetic basis of Ewing sarcoma, in particular the specific role of EWS/ETS fusion genes in tumorigenesis, relatively little is known about the cellular mechanisms that underlie metastasis and even less is known about the contribution of the local TME (8). Previous reports have shown that the bone TME specifically promotes metastatic progression of Ewing sarcoma (9) and that its ability to grow in bone is dependent on the osteolytic phenotype (10). Moreover, a hypoxic microenvironment promotes activation of metastasis-associated gene expression in tumor cells and enhanced metastatic progression in xenograft models (11, 12). In addition, gene expression profiling studies of primary localized tumor specimens demonstrated the important contribution of tumor stroma to relapse and patient survival (13). Thus, crosstalk between the Ewing sarcoma cells and their TME plays a key role in tumor progression. However, the Ewing sarcoma secretome is undefined, and its role in dictating ECM composition and tumor progression is yet to be elucidated.

We previously showed that activation of Wnt/beta-catenin in Ewing sarcoma cells induces transition to a more migratory cellular phenotype and enhances metastatic engraftment (14). In the current study, we have investigated whether activation of canonical Wnt signaling in Ewing sarcoma cells impacts on their secretion of ECM proteins. To address this, we combined mass spectrometry, proteomics, and

bioinformatics tools to define the Ewing sarcoma secretome. In addition, we determined if the secretome is impacted by exposure to exogenous Wnt3a, a canonical Wnt ligand abundant in the bone microenvironment (15, 16). Our results demonstrate that the Ewing sarcoma secretome is rich in ECM proteins, as well as IGF binding proteins. Activation of Wnt/beta-catenin modulates the secretion of ECM proteins, altering the local TME. These data provide novel insight into the nature of the Ewing sarcoma secretome and how it is altered by Wnt/beta-catenin signaling.

Results

Wnt-activated cells upregulate pathways involved in tumor: TME crosstalk.

To begin to understand the mechanisms through which upregulation of the Wnt/beta-catenin signaling axis contribute to metastatic phenotypes and poor outcomes in Ewing sarcoma, we performed pathways analysis on our previously reported RNA-sequencing study of Wnt/beta-catenin activated cells (14). In this previous study, 1157 transcripts were identified as being significantly induced by Wnt activation and 1221 were significantly induced in Wnt activation with potentiation by Rspondin2. Mapping of these transcripts against published signal transduction pathways using the Genomatix Pathway System tool revealed significant enrichment of signaling pathways that are known to play key roles in mediating tumor: TME interactions. In particular, signaling pathways that depend on crosstalk between receptors on tumor cells and proteins in the surrounding ECM were identified, including, integrin linked kinase, focal adhesion kinase, cadherin, angiogenesis, TGF beta and matrix metalloproteinase pathways (**Supplemental Figure 2.1A**). Additionally, gene ontology analysis of cellular

localization revealed significant enrichment of proteins found in focal adhesions, extracellular exosomes and the ECM (**Supplemental Figure 2.1B**). Thus, activation of canonical Wnt signaling in Ewing sarcoma cells leads to a significant increase in the transcription of genes that encode ECM proteins as well as other proteins that play key roles in tumor: TME interaction and crosstalk.

Identification of secreted proteins in Ewing sarcoma using label free mass spectrometry

ECM proteins in the local TME of solid tumors are most often secreted by tumor-associated fibroblasts and other non-malignant stromal cells (1). However, unlike many other solid tumors, non-tumor stromal cells are infrequently detected in Ewing sarcoma biopsies (13). This led us to speculate that the composition of the ECM in Ewing sarcoma may be largely determined by proteins that are secreted by the tumor cells themselves. Furthermore, in light of our observation that activation of canonical Wnt signaling induces transcription of ECM-encoding genes, we hypothesized that Wnt activation would alter the Ewing sarcoma secretome. In order to address this hypothesis, we used tandem mass spectrometry to define the secretomes of control and Wnt-activated Ewing sarcoma cells. TC32 and CHLA10 cells were chosen for these studies given that both are well-established Ewing sarcoma cell lines that grow as adherent monolayers on uncoated tissue culture plates. They were also found to generate less cell debris when exposed to serum free conditions than other Ewing sarcoma cell lines and to robustly activate TCF-dependent transcription in response to

Wnt3a (see **Figure 2.3** below). An outline of the experimental approach is summarized in **Figure 2.1**.

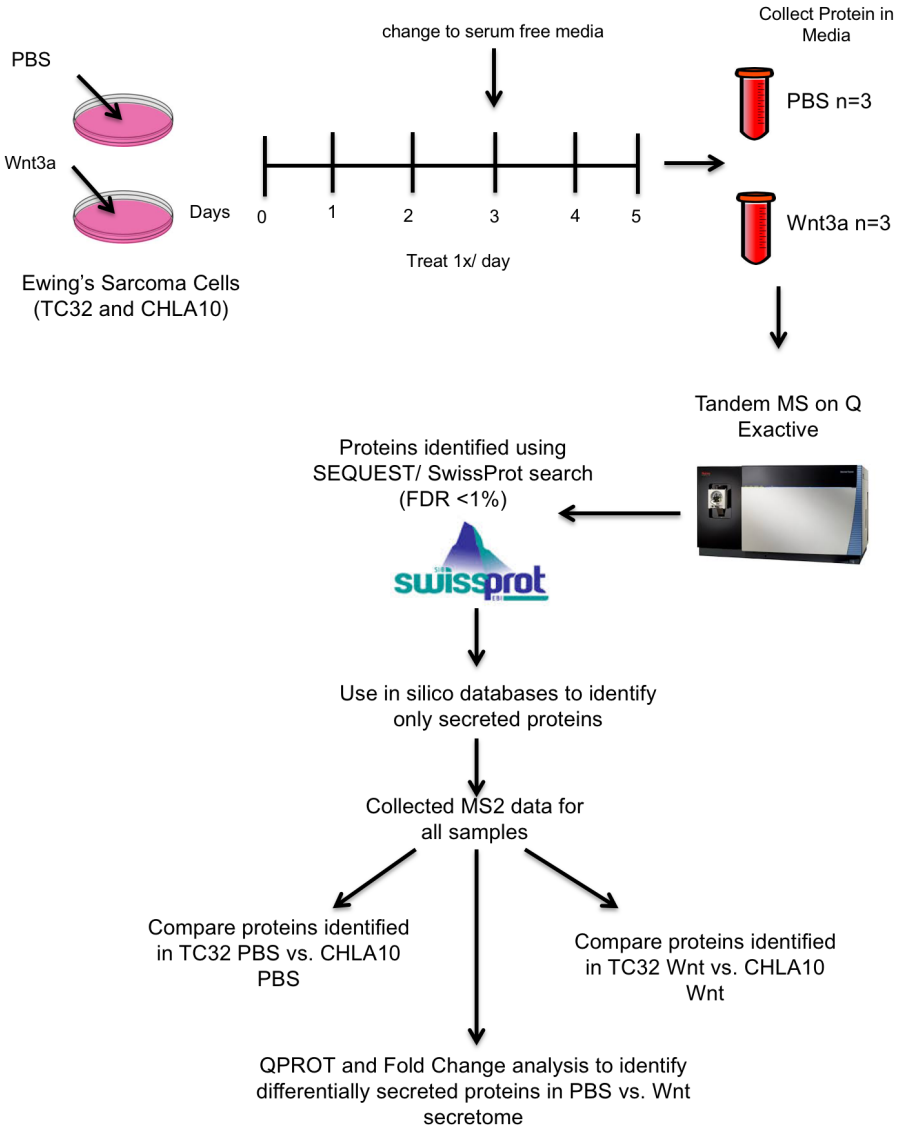


Figure 2.1 Identification of secreted proteins in Ewing sarcoma using label free mass spectrometry. Both TC32 and CHLA10 were treated with either vehicle (PBS) or recombinant human Wnt3a (100 ng/uL) once a day for 5 days prior to collection of media. Cell debris was monitored over time and only replicate cultures with excellent cell viability and minimal debris in the conditioned culture medium were collected for analysis. Media was collected gently to minimize cell rupture. All conditions were collected in triplicate and cell debris removed by centrifugation. Conditioned media was then concentrated and submitted for Tandem mass spectrometry and subsequent protein identification using Swissprot.

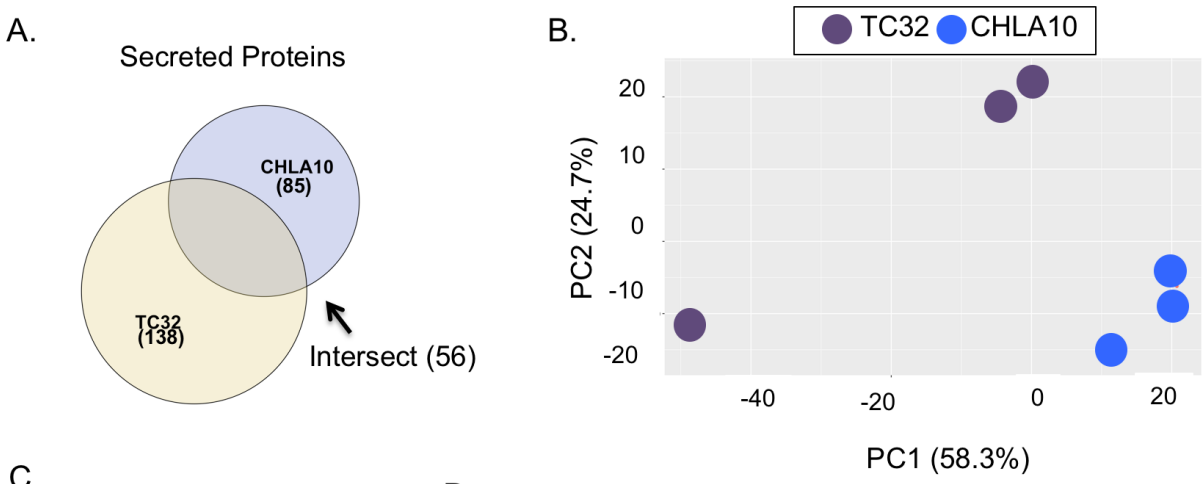
Conditioned media from control and experimental cells was analyzed by mass spectrometry, as described above, and the overall distribution of proteins identified across all conditions was first quantified. Since we used unlabeled mass spectrometry, all quantification was done using spectral counting and peptide-spectra-match (PSM) counts for each protein (17). The total number of proteins identified in the conditioned media was 2336 proteins for TC32 and 857 for CHLA10 cells (Supplemental Table 1). One caveat of using mass spectrometry for secretome analysis of conditioned media is that many proteins can be detected that are not considered part of the secretome. These proteins include those that are present in the media as a result of cell rupture, cell shedding, as debris from dying cells, or simply due to their overwhelming abundance in cultured cells. To address this key issue, and to minimize assignment of non-secreted proteins to the defined secretome, we gently collected conditioned media from the culture dishes without disrupting the cell monolayers and removed any cellular debris by centrifugation prior to mass spectrometry analysis. Next, we utilized four independent computational methods to analyze the mass spectrometry data to further ensure that the data represented secreted proteins. Four different analytic tools have been developed to rigorously assign identified proteins to the secretome: Human Protein Atlas, SignalP4.0, Phobius, and SPOCTOPUS (18-21). These tools predict the likelihood of a detected protein being secreted based on presence of a signal peptide sequence and absence of a transmembrane domain. Following application of these computational tools, 543 of 2336 (23%) proteins were identified as secreted proteins in the conditioned media of TC32 cells. For CHLA10 cells, 259 of the 857 (30%) proteins were classified as secreted. This proportion of secreted proteins in conditioned media is

consistent with prior reports and, thus, the reproducible protein data are likely to be highly representative of the Ewing sarcoma secretome (22, 23). Alignment of the secreted proteins to all proteins encoded by the human genome confirmed highly significant enrichment for proteins in the “extracellular region” and “extracellular space” compartments, with FDR of 6.03E-72 and 2.47E-68 respectively, validating their designation as secreted proteins. Finally, to ensure that the designation of proteins to the secretome was not biased in favor of either low- or high- abundance proteins, we compared the distribution of spectral counts from the list of secreted proteins to the list of all proteins identified. A similar range of counts was evident across both the total and the secreted protein lists, confirming that we had identified an unbiased list of proteins that belong to the Ewing sarcoma secretome (**Supplemental Figure 2.2**). This computationally-validated list of secreted proteins was used for all further analyses and validation studies (Supplemental Table 2).

Defining the baseline Ewing sarcoma secretome

Having defined the identities of secreted proteins in the conditioned media of cultured Ewing sarcoma cells we next sought to define the nature of these proteins in the two different cell lines in basal conditions. To our knowledge, the Ewing sarcoma secretome has not previously been characterized. Under basal conditions, 138 and 85 proteins were identified as being secreted into the media by TC32 and CHLA10 cells, respectively (**Figure 2.2A** and Supplemental Table 3). Principal component analysis of the data highlights that the secretome signature differs between the two cell lines, and that reproducibility between biologic replicates is high (**Figure 2.2B**). Interestingly,

however, over a third of proteins in the TC32 secretome and two-thirds of proteins in the CHLA10 secretome were shared, indicating that, despite some overall heterogeneity, there is evidence of a common secretome. Specifically, 56 proteins were reproducibly secreted by both Ewing sarcoma cell lines under basal, serum-free conditions (Supplemental Table 3). In addition, seven of the 20 most abundantly secreted proteins in each cell line, based on total PSM number, were shared (**Figure 2.2C**). Pathway analysis of the commonly secreted 56 proteins revealed enrichment for proteins involved in IGF transport, extracellular matrix organization, and elastic fiber formation (**Figure 2.2D**). This is especially interesting given that many insulin-like growth factor (IGF) family members are targets of the pathognomonic EWS-FLI1 fusion and that alterations in IGF signaling are characteristic of Ewing sarcoma (24, 25). In addition, fibronectin, a major component of the ECM, was robustly secreted by both cell lines demonstrating that a primary source of fibronectin in the local Ewing sarcoma TME is the tumor cells themselves (26, 27). Thus, from these data we conclude that modulators of IGF pathway signaling and ECM composition and organization are reproducibly and abundantly secreted by Ewing sarcoma tumor cells.



C.

Gene	TC32 PSM	CHLA10 PSM
HMCN 1	328	64
FN1	255	69
IGFBP2	123	324
FBN1	122	56
PXDN	115	69
FBN2	249	179
FBLN1	66	141

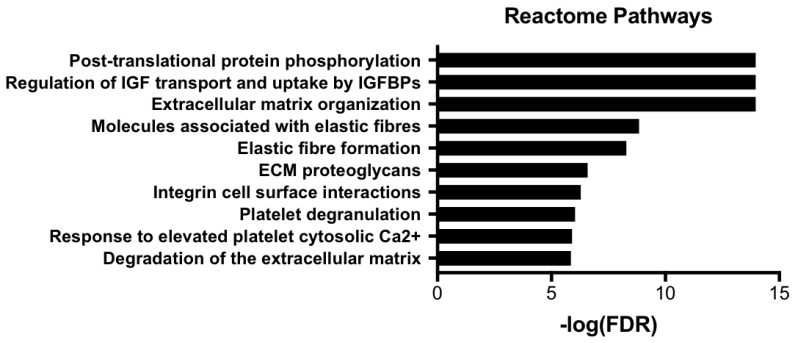


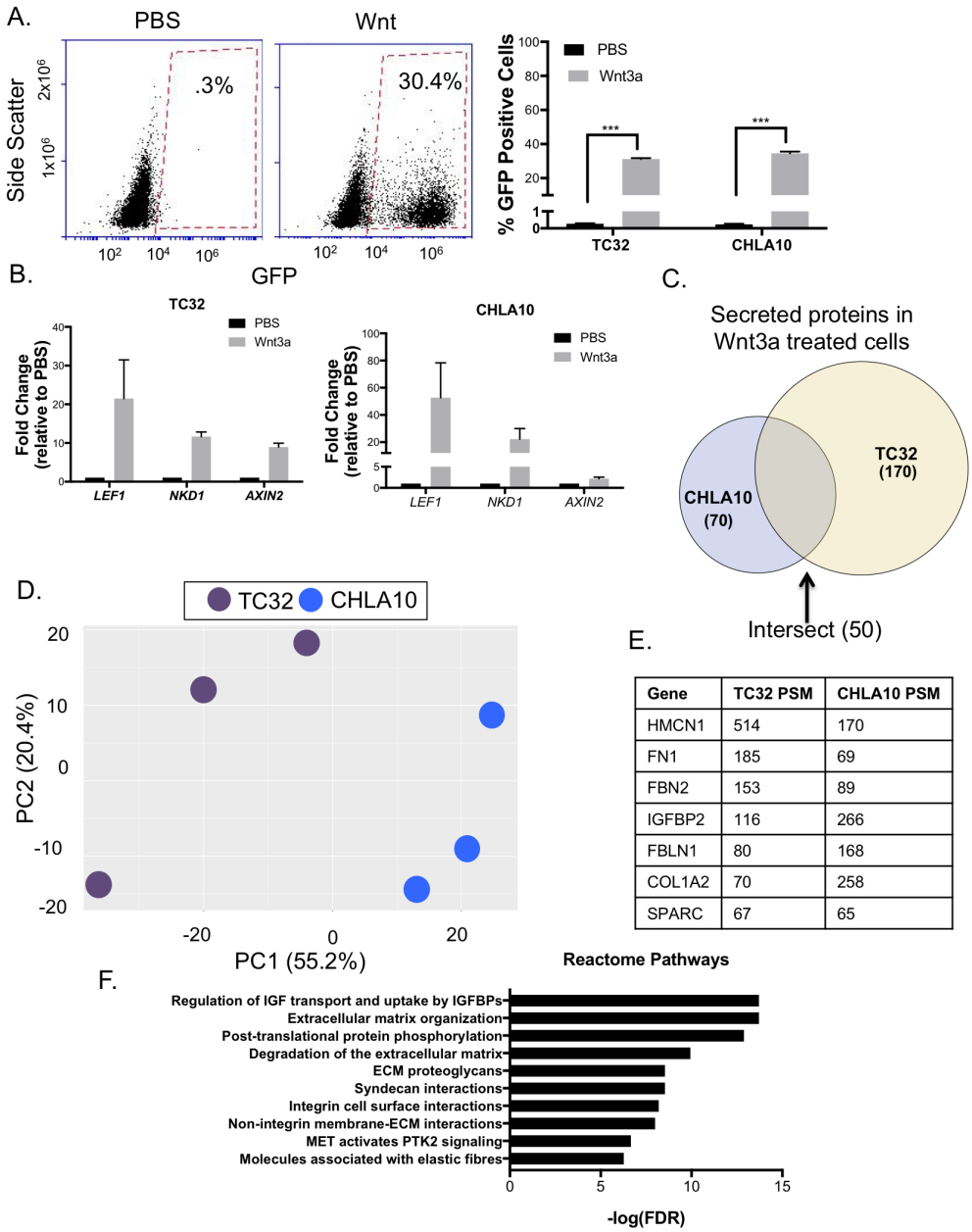
Figure 2.2 The baseline Ewing sarcoma secretome. A. Venn diagram showing the intersection between proteins identified at baseline in TC32 and CHLA10. 56 of the proteins were found in both samples. B. Principal component analysis of three TC32 and three CHLA10 replicates with vehicle treatment only. C. Total PSMs were added among all three replicates for each cell line and then ranked in descending order based on total PSM. 7 of the top 20 most abundant proteins were shared among both cell lines and are displayed here. D. Proteins present at baseline in both TC32 and CHLA10 were subject to pathway analysis using the reactome database. Top 10 pathways are shown here and all pathways have FDR < 1%.

Defining the Wnt-dependent Ewing sarcoma secretome

Having defined the basal secretomes of both TC32 and CHLA10 cells we next sought to assess the impact of canonical Wnt-activation. We previously observed that the response of Ewing sarcoma cells to exogenous Wnt3a ligand is highly

heterogeneous (14). Thus, we used flow cytometry analysis of TCF/LEF-GFP reporter cells, as previously described (14), to validate the extent of canonical Wnt signaling in Ewing sarcoma cells following the defined five-day exposure to Wnt3a. As shown, approximately one-third of cells in both TC32 and CHLA10 cell lines demonstrated activation of Wnt/beta-catenin-dependent transcription under the experimental conditions (**Figure 2.3A**). In addition, parallel analysis of mRNA from the same cultures confirmed robust and reproducible upregulation of the previously validated Wnt/beta-catenin target genes, *LEF1, NKD1*, and *AXIN2,* in Wnt-activated samples (**Figure 2.3B**).

Similar to the basal secretomes, the Wnt-activated secretome of TC32 contained more proteins than that of CHLA10. Whereas 170 secreted proteins were identified in the conditioned media of TC32 cells, 70 proteins were identified in the CHLA10 secretome. Principal component analysis again confirmed that the Wnt-activated secretomes differed between the two cell lines (**Figure 2.3D**). Nevertheless, 50 proteins were again shared (Supplemental Table 4), including IGFBP2, fibronectin, as well as other proteins that were also abundant in basal conditions (**Figure 2.3E**), Notably, two proteins that are integral to ECM composition and bone remodeling, collagen type 1 alpha 2 chain (COL1A2) (28) and secreted protein acidic and cysteine rich (SPARC) (29), were among the most abundant and reproducibly secreted under conditions of canonical Wnt activation (**Figure 2.3E**). Consistent with basal secretomes, analysis of the 50 shared proteins in Wnt-activated secretomes revealed enrichment for pathways involved in IGF signaling as well as ECM composition, organization, and degradation (**Figure 2.3F**).



Gene	TC32 PSM	CHLA10 PSM
HMCN1	514	170
FN1	185	69
FBN2	153	89
IGFBP2	116	266
FBLN1	80	168
COL1A2	70	258
SPARC	67	65

Figure 2.3 The wnt-dependent Ewing sarcoma secretome. A. Cells were transduced with 7TGP plasmid (plasmid #24305 from Addgene). Cells were treated for 5 days with Wnt3a (100 ng/uL) once a day prior to collection of cells for flow cytometry analysis. Analysis was run in triplicate and percentage of GFP positive cells are shown. Statistical significance was determined using student's t-test and *** = p-value <.0001. B. qRT-PCR validation of established Wnt targets in Ewing sarcoma. LEF1, NKD1, and AXIN2 were all upregulated in all replicates submitted for mass spectrometry. C. 170 proteins were identified to be secreted in TC32, 70 were identified in CHLA10, and 50 were identified in both cell lines. D. Principal component analysis of the Wnt-activated secretomes of each cell line. E. The total PSMs were added together for all three replicates of Wnt3a treatment and ranked in descending order based on total PSM. Of the top 20 proteins in each cell line, 7 were secreted by both cell lines. These proteins are shown here. F. Proteins present in both TC32 and CHLA10 were subject to pathway analysis using the reactome database. Top 10 pathways are shown here and all pathways have FDR < 1%.

Wnt activation in Ewing sarcoma cells promotes secretion of proteins that alter the composition and structure of the ECM.

Next, we assessed each cell line independently to determine if secretion of individual proteins changed in response to Wnt/beta-catenin activation. Total PSMs were used to determine differential expression between control and Wnt3a-treated samples. Differentially expressed proteins were defined as proteins that had a local FDR < 10% (computed using QPROT), a fold change score of >2 or <0.5, and detection of at least 2 unique peptides (see methods for further details) (30). Using these very stringent criteria, we identified significant changes in the secretomes of both cell lines following activation of canonical Wnt signaling. Specifically, TC32 cells showed altered secretion of 33 proteins, 27 of which were upregulated (**Figure 2.4A**). In CHLA10 cells, Wnt/beta-catenin activation led to altered secretion of 16 proteins with 14 of these having increased secretion (**Figure 2.4B**). Shared proteins with Wnt-dependent increases in secretion were Wnt3a, TNC, pro-enkephalin (PENK), and members of the proprotein convertase subtilisin/kexins family (PCSK2 and PCSK9). Given that Wnt3a was added to the media for these experiments, it is important to note that it was identified in both lists, confirming the validity of the analysis and serving as an internal quality control.

In order to better understand the nature of Wnt-dependent secretome, and to remove bias associated with *a priori* assignment of significance based on fold change, we performed Gene Set Enrichment Analysis (GSEA) on the ranked list of detected proteins and searched against the KEGG and Reactome databases (31). GSEA revealed the Wnt-dependent secretome to be enriched, in both cell lines, for proteins

that play key roles in ECM organizational structure and ECM: cell receptor interactions (**Supplemental Figure 2.3**). Notably, 5 of 27 proteins with increased secretion in Wnt/beta-catenin activated TC32 cells are structural proteins (COL1A1, COL1A2, COL3A1, COL5A1, and LAMA5), and another 9 have been published to play a role in collagen fibril and extracellular matrix organization (BGN(32), TGFBI(33-35), CTGF(36), MATN3(37), ADAM9 (38), MMP19(39), FMOD (28), LUM (28, 40) and TNC (41-43)). Thus, these data show that the secretome of TC32 cells is particularly responsive to canonical Wnt activation, and secretion of structural proteins that contribute to ECM organization and remodeling is significantly increased. Interestingly, the secretome of CHLA10 cells, an inherently highly metastatic cell line (44), contained abundant collagens at baseline and secretion was not further induced by Wnt stimulation (Supplemental Table 2).

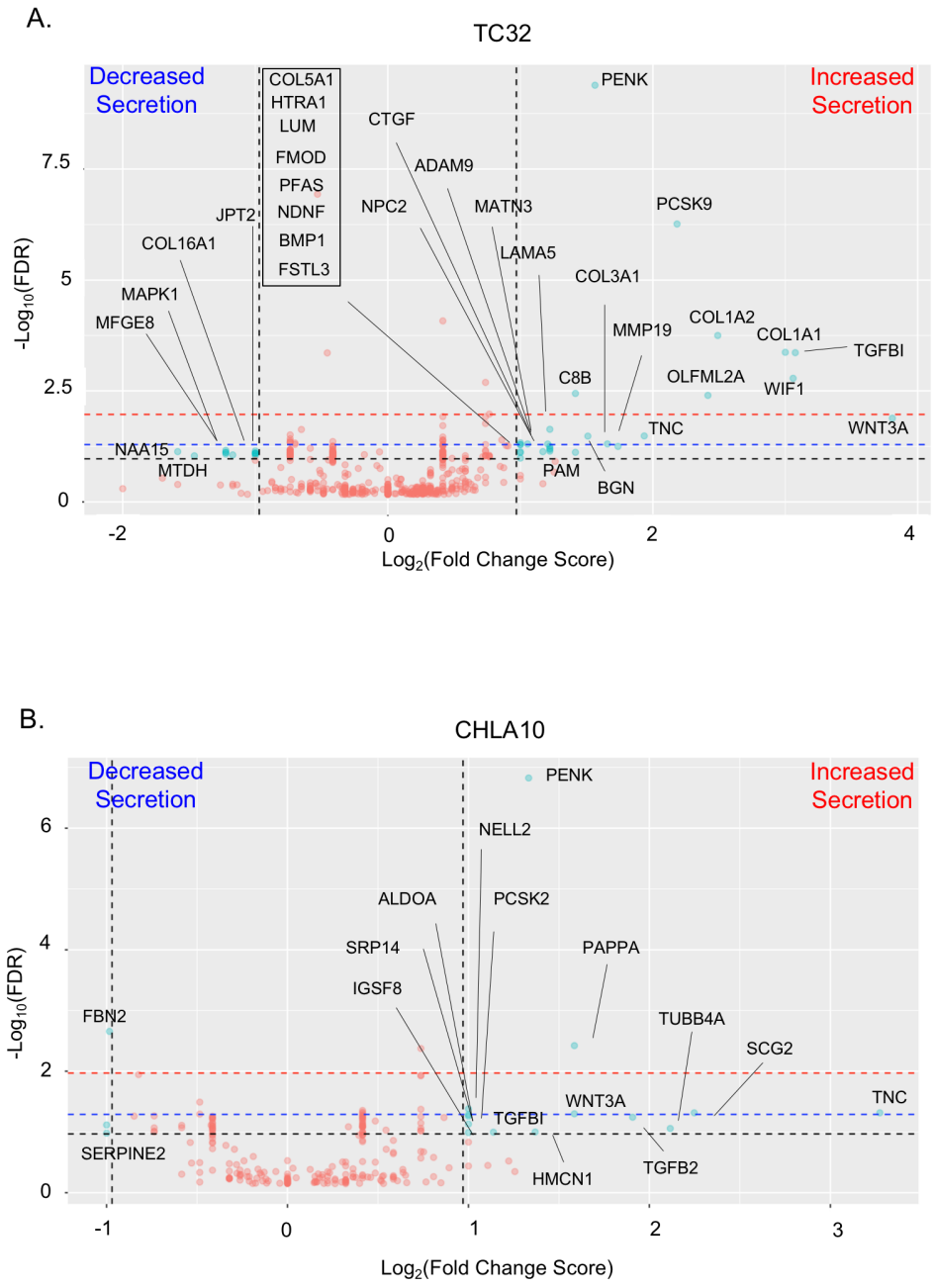


Figure 2.4 Stimulation with Wnt3a changes the Ewing sarcoma secretome. Volcano plots showing the Log_2(Fold Change Score) vs. -Log_{10}(localFDR). Red dots indicate proteins that were not differentially secreted and blue dots indicate proteins that were considered to be differentially secreted. Proteins that were considered differentially secreted have Fold Change Score >2 or <.5 and QPROT computed local FDR of differential expression <.1 (indicated by the black dashed lines). We also have noted that FDR <.05 results in 16 differentially secreted proteins in TC32 and 5 in CHLA10 (proteins that are above the blue dashed line). An FDR <.01 results in 8 proteins in TC32 and 2 in CHLA10 to be differentially secreted (proteins that are above the red dashed line).

To validate our findings, we used orthogonal methods to assess the impact of Wnt/beta-catenin activation on expression and secretion of TNC and collagen I. Both of these ECM proteins are well-established mediators of tumor engraftment and metastasis (43, 45). We previously showed that stimulation of Ewing sarcoma cells with

Wnt3a for 48 hours leads to a two-fold increase in expression of *TNC* mRNA (14). Analysis of cells following the five-day stimulation protocol used for secretome analysis confirmed robust upregulation of *TNC* mRNA to greater than five-fold (**Figure 2.5A**). Expression of the *COL1A1* transcript was also induced (**Figure 2.5B**). Next, we sought to validate whether activation of Wnt/beta-catenin in tumors *in vivo* is also associated with increased expression of these ECM protein-encoding genes. To achieve this we interrogated three independent, publicly available databases (13, 46, 47) and assessed correlations between expression of *LEF1* and *TNC* and *COL1A1* in primary patient tumors. We have previously shown that *LEF1* is a robust biomarker of Wnt/beta-catenin activation in Ewing sarcoma cells and tumors (14). As shown, expression of *TNC* was significantly positively correlated with *LEF1* in all datasets and *COL1A1* correlated with *LEF1* in two of the three patient cohorts (**Figure 2.5C**). As a control, we tested correlations of two other ECM protein-encoding genes, *FBLN1* and *LTBP3*, whose protein products were detected in the Ewing sarcoma secretome but were not impacted by Wnt activation (Supplemental Table 2). As shown, expression of neither of these genes correlated with *LEF1* in any of the three primary Ewing sarcoma datasets (**Figure 2.5C**). Thus, these data support the conclusion that, consistent with their response *in vitro*, activation of Wnt/beta-catenin in Ewing sarcoma cells *in vivo* leads to upregulated expression of the ECM protein-encoding genes *TNC* and *COL1A1*.

Finally, we sought to directly validate our mass spec findings that TNC and collagen I are secreted by Ewing sarcoma cells. To achieve this, we performed western blot on concentrated conditioned media collected from independent experiments. As shown, increased secretion of TNC was evident in the conditioned media of both TC32

and CHLA10 cells following Wnt3a stimulation (**Figure 2.5D**). Likewise, an increase in collagen I secretion was detected in the conditioned media of Wnt-stimulated TC32 cells (**Figure 2.5E**). Consistent with our mass spec results, Collagen I was readily detected in the media of Wnt-activated CHLA10 cells, but was not increased beyond the high levels that were already secreted under basal conditions (**Figure 2.5E**).

Together these studies confirm that activation of Wnt/beta-catenin signaling in Ewing sarcoma cells leads to increased expression and secretion of ECM proteins that have the potential to impact on tumor progression by altering tumor: TME crosstalk.

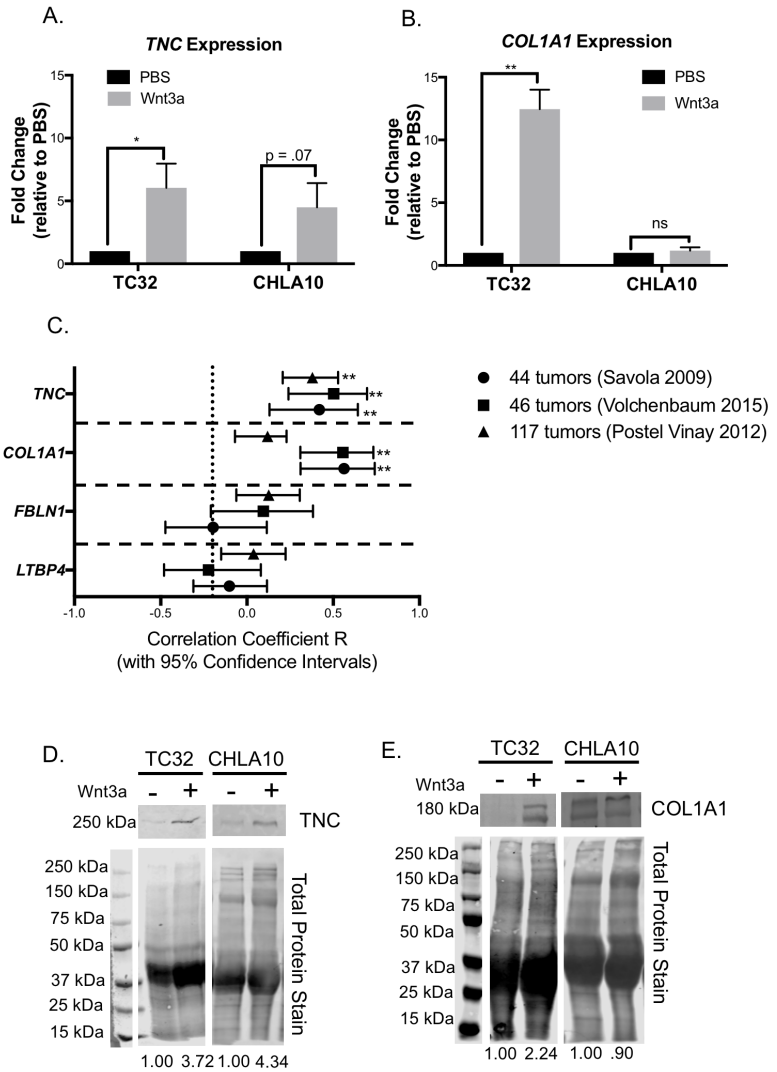


Figure 2.5 Activation of Wnt/beta-catenin is associated with high expression of *TNC* and *COL1A1* in Ewing sarcoma patients. A. qRT-PCR of *TNC* expression after 5 days of treatment with Wnt3a. B. qRT-PCR of *COL1A1* expression after 5 days of treatment with Wnt3a. C. Confidence intervals for pearson correlation of *LEF1* with *TNC, COL1A1, FBLN1*, and *LTBP3* from microarray analysis of three different patient cohorts, Volchenbaum 2015 (13), Postel Vinay 2012 (46), and Savola 2009 (47). The number of unique patient tumors in each dataset is shown (legend on right). D. Validation of increased secretion of TNC by Ewing sarcoma cells post Wnt3a treatment (western blot of conditioned media). Note that although TNC is expressed by Ewing sarcoma cells, its presence is not robustly detected in the secretome in the absence of Wnt3a stimulation. Statistical significance was determined using student's t-test with *= p-value < .05 and ** = p-value <.001. E. Validation of increased secretion of COL1A1 post Wnt3a treatment in TC32 and high basal levels in CHLA10.

Discussion

Secreted proteins play a role in every hallmark of cancer and are critical to tumor progression (6). Our data show that under basal, non-stimulated conditions, Ewing sarcoma cells secrete abundant proteins that play key roles in modulation of IGF signaling and the composition of the local tumor ECM. In addition, we find that, upon activation of Wnt/beta-catenin, Ewing sarcoma cells alter their secretomes in a manner that leads to increased secretion of matricellular and structural ECM proteins. We previously reported that activation of Wnt/beta-catenin signaling in Ewing sarcoma cells leads to an increase in expression of TNC, and that this is associated with enhanced engraftment and metastatic potential (14). In the current study we have confirmed that, not only is TNC expression upregulated, but that its secretion by tumor cells into the local TME is robustly increased. TNC interacts with FN1, another abundant ECM protein detected in our secretome analysis. TNC contains up to 17 FNIII repeats and is able to bind to the FNIII domain of FN1 through syndecan 4 (48). Thus, the increase in TNC secretion is expected to alter the physical interaction between TNC and FN1, resulting in altered TME structure. TNC has previously been reported to play a role in establishing the pre-metastatic niche in breast cancer, in addition to being highly expressed in the secretome of aggressive colorectal cancers (49, 50). Thus, these studies lend evidence to support the hypothesis that Wnt/beta-catenin dependent activation of TNC contributes to the metastatic phenotype of Ewing sarcoma by altering the local ECM.

Despite inter-tumor heterogeneity, our studies of two different cell lines revealed substantial overlap in secreted proteins. We particularly noted the presence of members

the IGF pathway. The IGF1-receptor is necessary for EWS-FLI1-induced malignant transformation and activation of IGF signaling contributes to tumor initiation and progression (24, 25, 51). We detected abundant secretion of IGF family members, specifically IGFBPs, in the Ewing sarcoma secretome, and the overall level of secretion did not change under conditions of Wnt/beta-catenin activation. Interestingly, however, Wnt/beta-catenin activation did lead to a reproducible increase in secretion of the IGFBP protease, pappalysin-1 (PAPPA) (Fold change scores 2.2 and 3.0 for TC32 and CHLA10 cells, respectively). PAPPA cleaves IGFBPs, thereby releasing IGF ligands, and promoting activation of the IGF signaling axis (52). Thus, a Wnt-dependent increase in PAPPA secretion could increase in the availability of IGF ligands in the local TME and activate IGF signaling in tumor cells. Despite its tremendous potential as a therapeutic target, and clear evidence of efficacy in preclinical models, the efficacy of IGF1-R targeted therapies in clinical trials has been mixed with response rates ranging from 10-14% in three independent clinical trials (53-55). To date, it is not known why some patients are so responsive to anti-IGF1-R strategies, nor how to identify them (56). Intriguingly, in a phase II study of 115 patients with relapsed or refractory Ewing sarcoma, 10 of 11 responses occurred in patients with bone tumors, suggesting that there may be something about bone tumor microenvironment that sensitizes tumors to IGF1 pathway targeted therapy (55). Given the high level of canonical Wnt ligands that are present in the bone, we speculate that activation of Wnt/beta-catenin in bone Ewing sarcoma cells may alter their secretome, resulting in an altered TME, promotion of IGF1 pathway activation, and enhanced sensitivity to anti-IGF approaches.

In addition to IGFBPs, FN1 and other proteins involved in elastic fiber formation were abundantly secreted by Ewing sarcoma cells. This is consistent with earlier reports that Ewing sarcoma cells synthesize fibronectin, collagens, and laminin (26, 27). Notably, in the Wnt-activated secretomes, we detected further abundance of proteins that contribute to the structure and composition of the ECM. In particular, secretion of collagens as well as other glycoproteins involved in mediating collagen fibril formation (i.e. TNC, BGN, TGFBI, LUM, MATN3, ADAM9, MMP19, and FMOD) was significantly increased in TC32 cells, a cell line in which canonical Wnt activation results in enhanced metastatic engraftment (14). Changes in the structural and biochemical properties of the ECM play fundamental roles in cancer progression (2). Not only does changing the biochemical properties lead to aberrant alterations in classical signaling pathways, such as TGF-beta and Wnt signaling (57, 58), but changes in synthesis, deposition, and degradation of ECM components, including collagen I, III, and V, are able to further promote tumor progression (59). Hayashi et al., recently reported that inhibition of canonical Wnt signaling in Ewing sarcoma cells resulted in an increase in metastasis-free survival in orthotopic bone tumor models and this was associated with decreased expression of *COL1A2* and *COL3A1* (60). Moreover, in the current study, COL1A1, COL1A2, COL3A1, and COL5A1 were abundant in CHLA10 secretomes, under both basal and Wnt-activated conditions. CHLA10 is an inherently metastatic cell line (44), irrespective of Wnt-activation, leading us to speculate that secretion of collagens by Ewing sarcoma cells may be a key determinant of their metastatic potential. Indeed, the propensity of undifferentiated soft tissue sarcomas to metastasize is, in part, dependent on modification and organization of collagens in the local TME

(61). Further experiments are now needed to understand the contribution of altered collagen secretion and organization to metastatic progression of Ewing sarcoma.

In summary, through an unbiased proteomics approach we have defined the Ewing sarcoma secretome, under both basal and Wnt/beta-catenin activated conditions. Our findings demonstrate that Ewing sarcoma cells secrete numerous proteins that modulate IGF signaling as well as ECM composition and structure, and that activation of the canonical Wnt pathway leads to increased secretion of ECM matricellular and structural components. These findings support the conclusion that activation of canonical Wnt signaling in Ewing sarcoma cells leads to changes in the local TME that have the potential to impact on tumor crosstalk and metastatic progression.

Materials and Methods

Cell lines

Ewing sarcoma cell line TC32 was maintained in RPMI 1640 media (Gibco) supplemented with 10% FBS (Atlas Biologicals) and 2mmol/L-glutamine (Life Technologies. CHLA10 was maintained in IMDM media (Fisher Scientific) supplemented with 20% FBS, 2mmol/L-glutamine, and 1X Insulin-Transferrin-Selenium (Gibco). Both cell lines are late passage cell lines and were obtained from the Children's Oncology Group (COG) cell bank (cogcell.org). Identity of all cell lines was confirmed by STR profiling and absence of mycoplasma was confirmed within 6 months of the experiments performed in this manuscript.

Biological annotation of the differentially expressed transcripts

In order to understand the biological functions of the transcripts that were differently expressed in Wnt3a treated samples compared to that of the baseline group, we used the Genomatics software suite. The signal transduction pathway enrichment analyses were done using the Genomatix Pathway System (GePS) tool (www.genomatix.de/) embedded in Genomatix software. GePS uses information extracted from public and proprietary databases to display canonical pathways or to create networks based on literature data. Cellular compartment was determined using DAVID 6.8 (updated October 2016). All genes identified to be upregulated by Wnt3a+/-RSPO2 in our previously published work ((14) GSE75859) were compared against the human genome. All cellular compartments shown have FDR < 1%.

Experimental Design and Statistical Rationale

Cells were either treated with vehicle (PBS, Life Technologies) or 100 ng/uL of recombinant Wnt3a (Wnt3a; R&D Systems). Cells were treated once a day for three days in media with full serum prior to changing to either RPMI or IMDM lacking FBS. Cells were cultured in 15 cm dishes for all experiments and media was collected from a single dish for each biologic replicate. Cells were then treated once a day for two additional days before media was collected. Protein in the media was concentrated using three kilodalton cutoff Amicon Ultra Centrifugal Filter Units (Fisher). Protein concentration was measured using the DC Protein Assay (Bio-Rad). Two cell lines were used for analysis with each condition. For each cell line, three replicates of control (vehicle treated samples) were compared against three replicates of Wnt3a treated

samples. All samples for each cell line were processed at the same time to account for batch effect.

Protein Identification by LC-tandem Mass Spectrometry

Equal amounts of proteins (50 ug) were used for processing. Cysteines were reduced with 10 mM DTT at 55 °C for 30 min and alkylated using 50 mM chloroacetamide (RT, 30 min). Digestion with 500 ng of sequencing grade, modified trypsin (Promega) was carried out overnight at 37 °C. Reaction was terminated by acidification with trifluoroacetic acid (0.1% v/v) and peptides were purified using SepPak C18 cartridge following manufacturer's protocol (Waters Corp). An aliquot of the resulting peptides (~2 ug) were resolved on a nano-capillary reverse phase column (Acclaim PepMap C18, 2 micron, 50 cm, ThermoScientific) using 0.1% formic acid/acetonitrile gradient at 300 nl/min (2-25% acetonitrile in 105 min; 25-40% acetonitrile in 20 min followed by a 90% acetonitrile wash for 10 min and a further 30 min re-equilibration with 2% acetonitrile) and directly introduced in to *Q Exactive HF* mass spectrometer (Thermo Scientific, San Jose CA). MS1 scans were acquired at 60K resolution (AGC target=$3e^6$, max IT=50ms). Data-dependent high-energy C-trap dissociation MS/MS spectra were acquired for the 20 most abundant ions (Top20) following each MS1 scan (15K resolution; AGC target=$1e^5$; relative CE ~28%). Data was analyzed using Proteome Discoverer (v2.1, Thermo Scientific). Proteins were identified by searching the data using Sequest HT against *Homo sapiens* (Swissprot, v2016-11-30, database includes 20,213 proteins) appended with common contaminants (100 total proteins). Search parameters included MS1 mass tolerance of 10 ppm and

fragment tolerance of 0.2 Da; the protease used was trypsin; two missed cleavages were allowed; carbamidimethylation of cysteine was considered fixed modification and oxidation of methionine, deamidation of asparagine and glutamine, phosphorylation of serine, threonine and tyrosine were considered as potential modifications. False discovery rate (FDR) was determined using Percolator and proteins/peptides with a FDR of ≤1% were retained for further analysis.

Identification of Secreted Proteins

In order to gather more information about the nature of the identifications, the predicted proteins were initially mapped to the Human Protein Atlas (HPA) Secretome and Proteome (18). HPA maintains a list of genes and proteins annotated as potentially secreted based on several inputs, including results from 3 bioinformatics applications (SignalP (19), Phobius (20), and SPOCTOPUS (21)) and one large-scale study (18). Proteins found in any of these lists were then classified as secreted and subject to further analysis.

Statistical Analysis and Interpretation of Results

Differential protein expression of proteins identified to be secreted in both vehicle and Wnt3a treated samples were analyzed by QPROT as described previously in (30). QPROT is developed specifically for modeling protein abundances in label free proteomics estimated using either MS1 intensity or the number of MS2 peptide-spectral-matches (PSMs). Proteins were considered to be statistically significant if they had fold change scroe >2 or < .5 and local FDR as computed by QPROT of < 10%. The fold

change score was calculated by averaging the total PSMs across all replicates in Wnt plus another factor (in this case we used 1) divided by the average PSMs across all replicates in PBS plus the same factor. Any proteins that had <2 unique peptides, and were only identified in 1 of 6 replicates, were not considered differentially secreted. Gene set enrichment analysis (GSEA) was conducted using the GSEA v2.1.0 software (31). Gene ontology for secreted proteins of non-Wnt activated cells was performed using DAVID 6.8 with the human genome as the background. Pathway analysis for the baseline secretome and Wnt-activated secretome was done using the Reactome pathway knowledgebase (62).

Quantitative real-time PCR

Total RNA was extracted from cells at the same time as protein collection 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 geometric mean of the two housekeeping genes GAPDH and HPRT. The following primers were used: LEF1 forward – 5’TGGATCTCTTTCTCCACCCA3’ and reverse – 5’CACTGTAAGTGATGAGGGGG3’, NKD1 forward – 5’TCGCCGGGATAGAAAACTACA3’and reverse – 5’CAGTTCTGACTTCTGGGCCAC3’, AXIN2 forward – 5’AAGTGCAAACTTTCGCCAAC and reverse – 5’ACAGGATCGCTC CTCTTGAA, HPRT forward - 5’TGACACTGGCAAAACAATGCA3’ and reverse –

5’GGTCCTTTTCACCAGCAAGCT3’, GAPDH forward – 5’TGCACCACCAACTGCTTAGC3’ and reverse – 5’GGCATGGACTGTGGTCATGAG3’, TNC forward – 5’GCAGCTCCACACTCCAGGTA3’ and reverse – 5’TTCAGCAGAATTGGGGATTT3’, and COL1A1 forward – 5’CTGGACCTAAAGGTGCTGCT3’ and reverse – 5’GCTCCAGCCTCTCCATCTTT3’.

Flow Cytometry

Stably transduced 7xTcf-eGFP (7TGP) (14) TC32 and CHLA10 cells were stimulated with Wnt3a (100ng/mL) once a day for 5 days prior to collecting cells to measure GFP fluorescence. Fluorescence was measured and quantified on an Accuri C6 cytometer (BD Biosciences).

Western Blots

Western blot analysis was performed using the Bio-Rad Mini-PROTEAN Tetra System. Following transfer, nitrocellulose membranes were blocked in 5% BSA in TBS-T for 1 hour. Membranes were washed once with TBS-T and then incubated overnight at 4^{o} C with either mouse-anti human Tenascin C (1:500, Sigma T2551) or rabbit-anti Collagen 1 (1:1000, Abcam ab34710). Membranes were then washed three times in 1X TBS-T for 5 minutes each and then incubated with secondary antibody IRDye 800CW goat anti-mouse (1:10,000, LiCor) or IRDye 800CW goat anti-rabbit (1:10,000, LiCor) for 1 hour. They were then washed two times with 1X TBS-T and once with 1X TBS for 5 minutes prior to scanning the membrane using the LiCor Imaging system.

Gene expression in Ewing sarcoma patient tumors

Pearson correlation coefficients of gene expression and associated confidence intervals in primary patient tumors were determined using three previously reported, independent datasets: GSE 63157 (13), GSE 34620 (46), and GSE 17679 (47). Only data from patient tumors were extracted from the GSE17679 dataset and the average value for duplicate assays from each of 44 unique patient tumors was used.

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Chapter 3: Cooperation between canonical Wnt and TGF-beta pathways promotes sarcoma angiogenesis

Summary

Local and metastatic progression of solid tumors depends on crosstalk between tumor cells and the tumor microenvironment (TME), including both stromal cells and the extracellular matrix (ECM). We recently showed that high Wnt/beta-catenin activity in Ewing sarcoma correlates with diminished patient survival and that canonical Wnt signaling alters the tumor secretome, influencing ECM protein composition. In light of this, we investigated the hypothesis that Wnt/beta-catenin supports tumor progression by modulating tumor: TME crosstalk. Our results reveal that, in discrete tumor cell sub-populations, beta-catenin activation sensitizes cells to TGF-beta ligands, resulting in canonical Wnt-induced, TGF-beta-dependent upregulation of TGF-beta targets, including numerous AngioMatrix proteins that are known to alter the TME to promote angiogenesis. Studies of Ewing sarcoma models, *in vitro* and *in vivo,* as well as primary tumor tissues, confirm that a direct relationship exists between beta-catenin activation in tumor cells and angiogenesis in the local TME. Mechanistically, this is due, in part, to tenascin C-mediated promotion of endothelial cell proliferation. Thus, functional cooperation between canonical Wnt and TGF-beta signaling in Ewing cells induces secretion of pro-angiogenic factors. Together, these studies illustrate the critical

contribution of tumor cell heterogeneity and tumor: TME crosstalk to sarcoma progression.

Introduction

Tumor growth and metastatic progression is dependent on the local “soil” or tumor microenvironment (TME), as posited in the seed and soil hypothesis put forth by Stephen Paget in 1889 (1). Together, the tumor and the local TME, comprised of secreted proteins, infiltrating non-cancerous cells, and blood vessels, create an ecosystem that is conducive to supporting tumor growth. Creation of this supportive niche is dependent on composition and structure of the local extracellular matrix (ECM) (1). Recent findings from our group and others have shown that activation of the Wnt/beta-catenin pathway in Ewing sarcoma is associated with alterations in the local TME and transition to a metastatic phenotype (2-5). Additionally, gene expression profiling of primary, localized tumors supports a role for the TME in driving metastasis, revealing the critical contribution of the TME to clinical outcomes in Ewing sarcoma (6).

Ewing sarcomas are aggressive bone and soft tissue tumors with peak incidence in adolescents and young adults (7). Pathologically, they are defined by an undifferentiated cellular histology and characteristic chromosomal translocations that give rise to EWS-ETS fusion proteins, the most common of which is EWS-FLI1 (8). EWS-ETS fusions are critical for the initiation, maintenance, and progression of this tumor, and they often represent the sole identifiable genetic lesion (9-11). Nevertheless, although most primary Ewing sarcoma tumors harbor quiet genomes, the clinical outcomes differ widely among patients and nearly a third of patients ultimately succumb

to disease recurrence and metastatic progression (7, 9). To date, the mechanisms that contribute to disease progression remain poorly understood, and this gap in knowledge has impeded the development of novel therapeutic strategies and prognostic biomarkers (12, 13).

The canonical Wnt/beta-catenin signaling pathway is critical for embryogenesis and normal development, but is co-opted in numerous tumors to promote proliferation, survival, and metastasis (14). Extracellular canonical Wnt ligands bind to cell surface receptors, resulting in activation of an intracellular signaling cascade that culminates in stabilization and nuclear localization of beta-catenin. Once in the nucleus, beta-catenin partners with the TCF/LEF family of transcription factors to activate transcription of cell-type and context-dependent target genes that alter cellular phenotypes (14). In Ewing sarcoma cells, activation of the canonical Wnt-dependent transcriptome promotes transition from a proliferative to a more migratory and metastatic state, a phenotypic switch that is associated with tumor cell-autonomous changes in the cytoskeleton (5, 15). Paradoxically, this activation of Wnt/beta-catenin partially antagonizes the EWS-ETS-dependent transcriptome, suggesting that these competing transcriptional programs modulate cell plasticity between non-motile and motile cell states (5). In addition, we recently reported the potential for tumor cell non-autonomous functions for Wnt/beta-catenin signaling in Ewing sarcoma progression. We showed that activation of the canonical Wnt pathway induces secretion of ECM and ECM associated proteins that alter the composition of the local TME. Specifically, we reported increased secretion of Tenascin C (TNC), a matricellular protein reported to mediate metastasis in Ewing

sarcoma (5), breast cancer (16), colorectal cancer (17), and many other solid tumors (18).

The exact mechanism through which Wnt dependent changes in the transcriptome and secretome contribute to changing tumor: TME crosstalk and metastatic progression remains to be elucidated. In the current study, we investigated the functional consequences of Wnt/beta-catenin activation on tumor: TME crosstalk. Our results have uncovered a previously unknown role for cooperation between canonical Wnt and TGF-beta pathways in mediating a pro-angiogenic TME in Ewing sarcoma.

Results

Beta-catenin activation correlates with extracellular matrix and EMT gene signatures in primary tumors

We previously showed that high expression of *LEF1,* a robust biomarker of Wnt/beta-catenin pathway activation in Ewing sarcoma, was associated with worse outcomes in patients who presented with localized disease (GSE63157) (5). These observations, together with our finding that Wnt/beta-catenin activation upregulates secretion of ECM proteins (3), led us to hypothesize that there may be a direct relationship between canonical Wnt pathway activation in Ewing tumor cells, the tumor microenvironment (TME), and disease progression. To address this hypothesis, we first evaluated whether a correlation exists in primary tumors between *LEF1* expression and expression of genes that are important in tumor: TME interactions. Using a stringent cutoff of pearson correlation $r > 0.5$, we identified 376 and 688 genes that were

positively correlated with *LEF1* in two independent patient cohorts of 46 (6) and 117 (19) tumors, respectively (**Table 3.1**). Interrogation of these gene lists using the bioinformatics tool DAVID (20) revealed a striking enrichment for biologic processes involved in ECM organization, cell adhesion, and angiogenesis (**Table 3.1**). Moreover, unbiased gene set enrichment analysis (GSEA) of both datasets in their entirety (21), confirmed highly statistically significant and reproducible correlations between *LEF1* and gene programs and signaling pathways that are crucial for tumor: TME interactions (**Figure 3.1**). Interestingly, in both independent patient cohorts, the top hit in the GSEA analysis ranked on *LEF1* correlation was epithelial mesenchymal transition (EMT). Classically, in tumor biology EMT is limited to carcinoma cells and is induced in these epithelial-derived cells by the TGF-beta pathway (22). Significantly, however, recent studies have suggested that, despite their mesenchymal origins, some sarcoma cells exist in a metastable state, allowing for transition between more epithelial-like and more mesenchymal-like phenotypes (23). We therefore investigated whether the phenotypic plasticity of Ewing sarcoma cells, that we previously described in response to Wnt/beta-catenin activation (5), is influenced by TGF-beta signaling.

Wnt/beta-catenin leads to de-repression of the *TGFBR2* locus and sensitizes cells to TGF-beta pathway activation

Prior studies have shown that Ewing sarcoma cells are unresponsive to TGF-beta ligand induced pathway activation as a consequence of EWS-FLI1-mediated repression of the core pathway receptor, *TGFBR2* (24). We previously showed that activation of the Wnt/beta-catenin pathway antagonizes EWS-FLI1 activity, resulting in

Term	Count	Fold Enrichment	p-value	FDR
GSE63157 (46 tumors)				
GO:0001525~angiogenesis	38	7.61	7.1E-22	1.2E-18
GO:0030198~extracellular matrix organization	35	7.97	8.0E-21	1.4E-17
GO:0007155~cell adhesion	46	4.48	4.4E-17	7.6E-14
GO:0050900~leukocyte migration	25	9.15	2.4E-16	3.9E-13
GO:0030574~collagen catabolic process	19	13.26	1.8E-15	3.1E-12
GO:0030168~platelet activation	21	8.16	9.7E-13	1.7E-09
GO:0007229~integrin-mediated signaling pathway	17	7.67	5.8E-10	1.0E-06
GO:0048010~vascular endothelial growth factor receptor signaling pathway	14	8.68	6.0E-09	1.0E-05
GO:0022617~extracellular matrix disassembly	14	8.23	1.2E-08	2.1E-05
GO:0045766~positive regulation of angiogenesis	16	6.21	4.1E-08	7.1E-05
GO:0001938~positive regulation of endothelial cell proliferation	12	7.77	3.4E-07	5.9E-04
GO:0006954~inflammatory response	27	3.18	4.3E-07	7.5E-04
GSE34620 (117 tumors)				
GO:0030198~extracellular matrix organization	57	7.10	4.8E-32	8.7E-29
GO:0007155~cell adhesion	80	4.25	2.7E-28	4.9E-25
GO:0030574~collagen catabolic process	28	10.68	3.9E-21	7.0E-18
GO:0001525~angiogenesis	46	5.03	2.5E-19	4.6E-16
GO:0050900~leukocyte migration	32	6.40	1.1E-16	2.0E-13
GO:0006955~immune response	55	3.19	7.4E-14	1.3E-10
GO:0030335~positive regulation of cell migration	35	4.64	1.1E-13	2.1E-10
GO:0007229~integrin-mediated signaling pathway	24	5.92	8.4E-12	1.5E-08
GO:0030199~collagen fibril organization	16	10.01	1.3E-11	2.4E-08
GO:0022617~extracellular matrix disassembly	21	6.74	1.7E-11	3.0E-08
GO:0006954~inflammatory response	47	3.03	3.8E-11	6.9E-08
GO:0007165~signal transduction	95	2.00	1.1E-10	2.0E-07

Table 3.1. *LEF1* correlated genes (Pearson r>0.5) in primary Ewing tumor biopsies are highly significantly enriched for biologic processes involved in tumor: TME interactions, including extracellular matrix organization, cell adhesion and angiogenesis. (Top 10 DAVID)

de-repression of many EWS-FLI1 repressed genes (5). This led us to hypothesize that activation of canonical Wnt signaling in Ewing cells might lead to induction of *TGFBR2*, thereby sensitizing them to TGF-beta. To address this, we first measured *TGFBR2* in a panel of Ewing sarcoma cell lines and confirmed expression to be very low relative to A549 lung adenocarcinoma cells (**Figure 3.2A**). Consistent with our hypothesis, interrogation of previously generated RNA-sequencing data (5) showed that the *TGFBR2* transcript increased in Ewing sarcoma cells following exposure to Wnt3a conditioned media, and this was potentiated by the addition of R-spondin2 (**Figure 3.2B**). We validated this finding in four different Ewing cell lines, wherein a reproducible

increase in *TGFBR2* expression was observed following exposure to recombinant

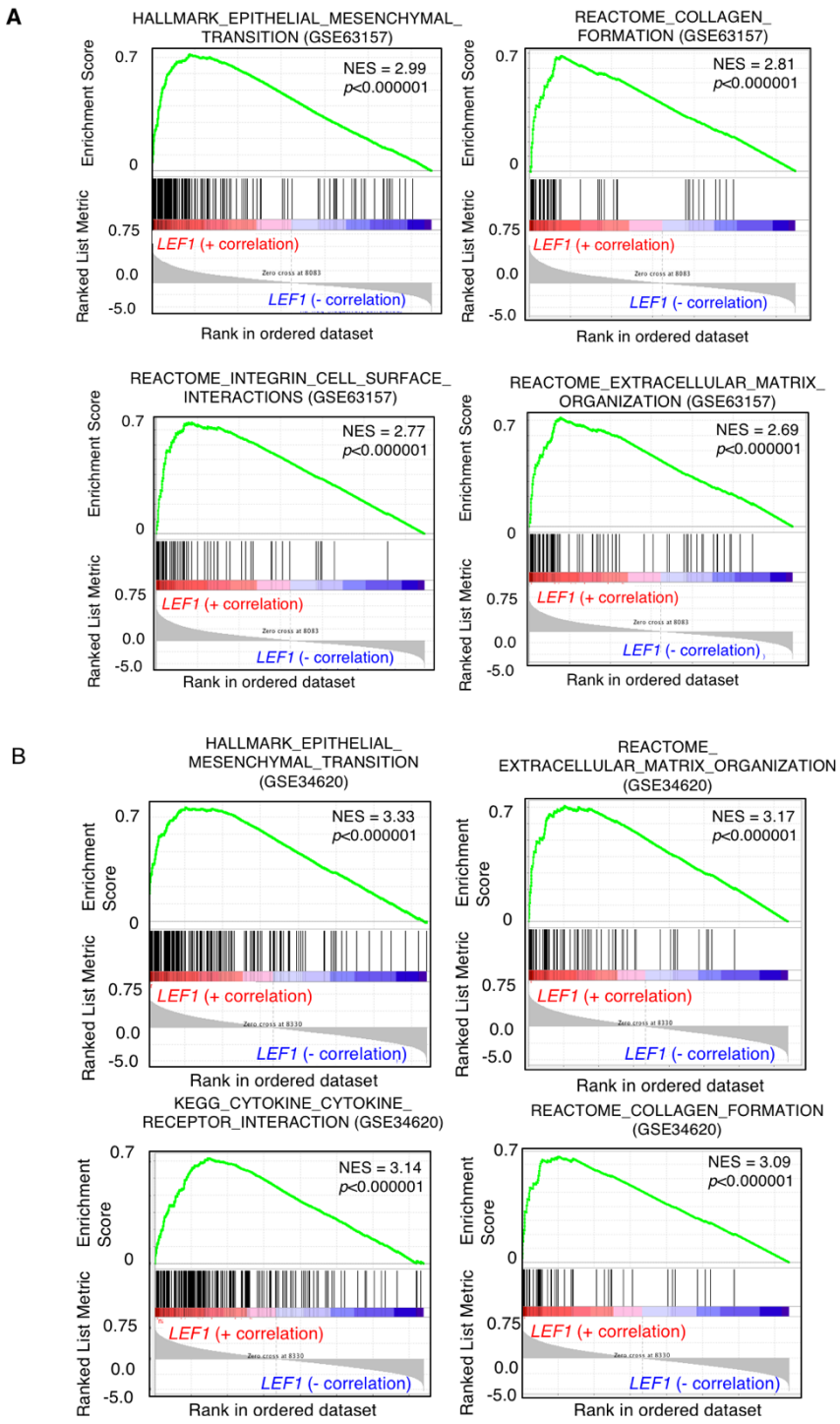

Figure 3.1. Beta-catenin activation correlates with extracellular matrix and EMT gene signatures in primary tumors. GSEA of whole transcriptome data from two independent primary tumor cohorts reveals genes that are highly correlated with *LEF1* expression are enriched for extracellular matrix and EMT associated gene signatures. Top 4 most enriched gene sets are shown in (A) for GSE63157; N=46 tumors, and in (B) for GSE34620; N=117 tumors.

Wnt3a (**Figure 3.2C**). To assess whether beta-catenin activation is also associated with *TGFBR2* de-repression *in vivo,* we assessed the relationship between its expression and that of *LEF1* in primary tumors. As shown, *LEF1* expression positively and significantly correlated with *TGFBR2* expression in both patient cohorts (**Figure 3.2D & E**). Thus, activation of canonical Wnt signaling in Ewing sarcoma cells leads to increased expression of *TGFBR2*.

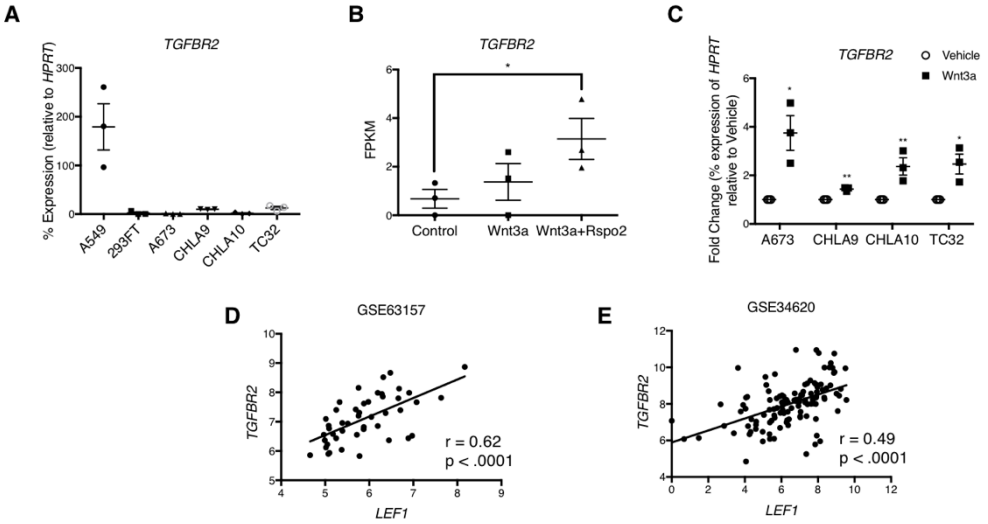

Figure 3.2. Wnt/beta-catenin leads to de-repression of the *TGFBR2* locus. A. qRT-PCR analysis of baseline *TGFBR2* expression in Ewing sarcoma (A673, CHLA9, CHLA10, TC32), embryonic kidney cells (293FT) (Negative control), and lung adenocarcinoma (A549) cell lines (Positive control) (N=3). B. RNA-seq analysis of *TGFBR2* expression Ewing sarcoma cells (CHLA25) following exposure to Wnt3a conditioned media or Wnt3a+ R-spondin2 (GSE75859) (N=3). C. qRT-PCR of *TGFBR2* expression in Ewing sarcoma cell lines following exposure to vehicle or recombinant Wnt3a (N=3). P-values were determined using student's t-test. *p<0.05, ** p<0.005 D. & E. Expression of *TGFBR2* is correlated with *LEF1* in primary tumor biopsies. X and Y axes represent log2(signal intensity) of labeled gene. Correlation was determined using Pearson r calculation. D: N=46 tumors, E: N=117 tumors.

Having confirmed that Wnt/beta-catenin activation leads to de-repression of *TGFBR2*, we next sought to test whether these cells activate the TGF-beta signaling

pathway in response to TGF-beta ligands. Previously it had been reported that TGFB1 is unable to induce TGF-beta pathway activation in Ewing sarcoma cells (24). In keeping with this observation, phosphorylation of SMAD3 (pSMAD3), a well-established member of the canonical TGF-beta signaling cascade, was robustly induced by TGFB1 in A549 cells but was only minimally or not induced at all in Ewing sarcoma cells (**Figure 3.3A**). Next, we pretreated cells with either vehicle or Wnt3a for 24 hours prior to addition of TGFB1, expecting that we would see an increase in pSMAD3 in Wnt3a-treated cells. However, in these studies of bulk populations, no appreciable change in pSMAD3 activation was observed (**Supplemental Figure 3.1A**). We have previously shown that heterogeneity in the Wnt3a response exists in Ewing sarcoma and that only discrete sub-populations of cells activate the beta-catenin/TCF/LEF-transcriptional axis in response to canonical Wnt ligand (5). Therefore, we hypothesized that de-repression of *TGFBR2* and sensitization to TGFB1 would be limited to the Wnt-responsive cells. To test this, we first sorted cells into Wnt-responsive (GFP Positive) and Wnt-unresponsive (GFP Negative) cells on the basis of a TCF/LEF-reporter, as previously described (5) (**Supplemental Fig. 3.1B**). Wnt responsive and Wnt unresponsive cells were then pretreated with vehicle or Wnt3a for 24 hours prior to treatment with TGFB1. As shown, pSMAD3 was induced by TGFB1 in the Wnt responsive cells but not in the Wnt-unresponsive population in the Ewing sarcoma cell line CHLA10 (**Figure 3.3B**). To validate this finding of differential TGF-beta sensitivity in the two sub-populations we used an orthogonal and more sensitive bioluminescent reporter assay. TCF/LEF-GFP reporter cells were transduced with a Smad binding element (SBE) – Luciferin reporter, sorted for GFP following Wnt3a stimulation, and then treated with TGFB1 prior to

measuring luciferase activity. In keeping with pSMAD3 western blot assays, TGFB1 led to a greater increase in luciferase activity in Wnt-responsive than Wnt-unresponsive cells, although the extent of these responses varied both within and between cell lines (**Figure 3.3C**). Thus, activation of Wnt/beta-catenin signaling in heterogeneous subpopulations of Ewing sarcoma cells leads to derepression of *TGFBR2* and this primes tumor cells to respond to TGF-beta ligands.

Given the evidence of heterogeneity that we observed in both the Wnt and TGF-beta responses across experiments and cell lines, we turned to a fluorescence-based dual reporter system to evaluate Wnt/beta-catenin and TGF-beta dependent transcriptional activation at the level of individual cells. For these experiments, Ewing sarcoma cells expressing the TCF/LEF-GFP reporter were transduced to co-express a TGF-beta SMAD binding element reporter (SBE-mCherry; kind gift of Dr. Elaine Fuchs) (25). Dual reporter cells were pretreated with vehicle or Wnt3a prior to treatment with TGFB1 and then subjected to flow cytometry. Interestingly, even in the absence of Wnt3a, TGFB1 induced mCherry expression in 0.5 – 8% of cells, revealing that small numbers of TGF-beta responsive cells are present at baseline (**Figure 3.3D & E**). Pre-treatment with Wnt3a led to activation of GFP in 4.5-80% of cells, depending on the cell line, and 1.5-10% of these GFP+ cells were mCherry+ following TGFB1 treatment (**Supplemental Figure 3.1C**). Consistent with our prior studies, Wnt3a led to activation of the TCF/LEF-GFP reporter in 4.5-80% of cells, depending on the cell line, and with the exception of TC32 cells, Wnt3a alone did not alter the frequency of mCherry+ cells. In TC32 cells, addition of Wnt3a alone resulted in induction of a population of GFP+/mCherry+ cells indicating that autocrine TGF-beta ligands were able to activate

TGF-beta transcriptional activity in Wnt-responsive TC32 cells (**Figure 3.3E**). Provision of both ligands in the media resulted in an increase in mCherry+ cells in all cell lines, comprising either single and/or dual positive cells, and mCherry activity was blocked by exposure of cells to SB505124. Exposure of cells to SB505124, a TGF-beta inhibitor, decreased the frequency of mCherry+ cells, validating that mCherry expression was due to TGF-beta pathway activation and not leakiness of the reporter (**Figure 3.3D & E**).

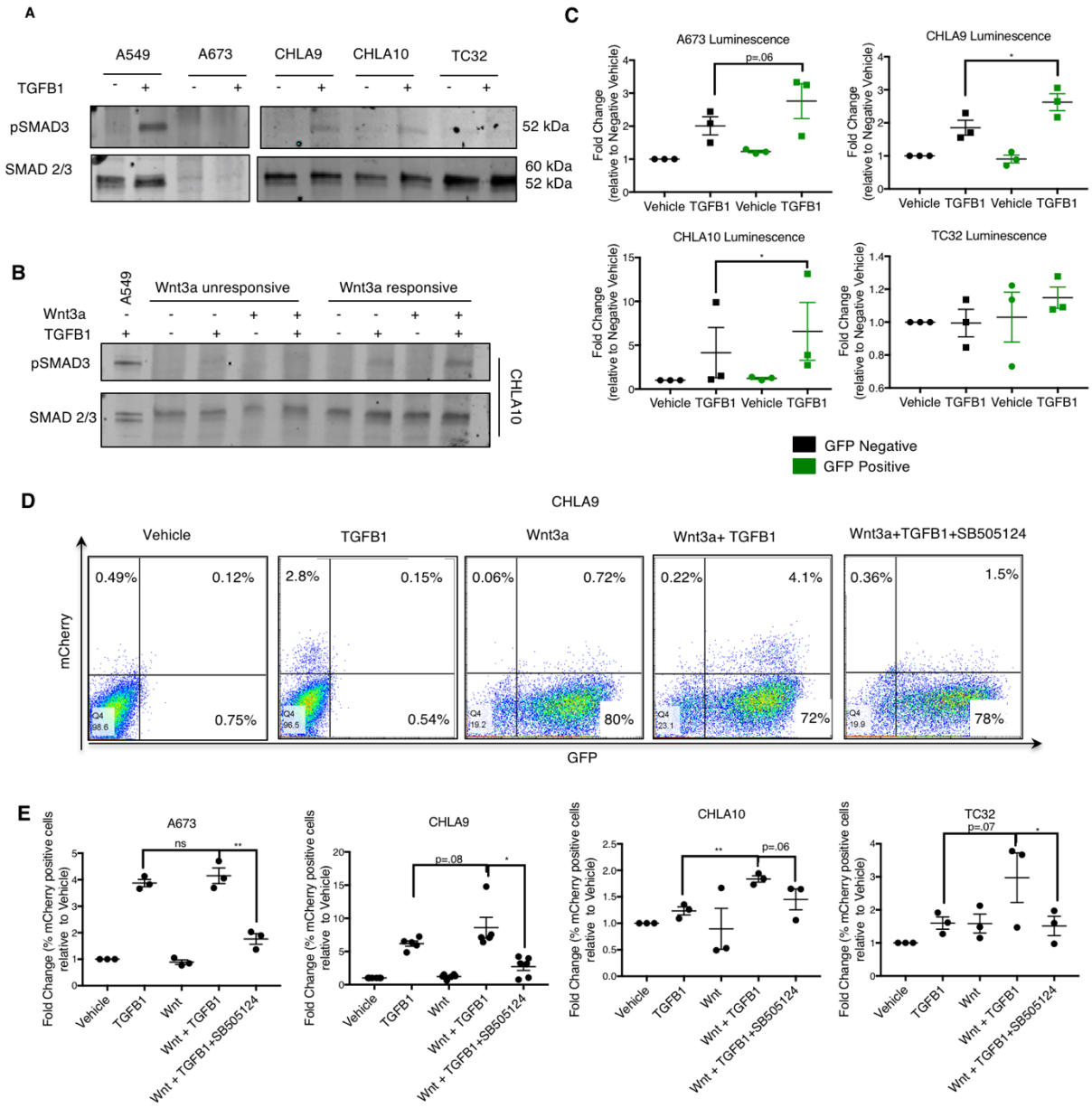

Figure 3.3. Wnt/beta-catenin activation sensitizes cells to TGF-beta. A. Western blot of pSMAD3 expression in Ewing sarcoma and A549 (positive control) cells at baseline and following exposure to TGFB1. B. Ewing sarcoma cells were treated with vehicle or Wnt3a and sorted into Wnt responsive (GFP Positive) and Wnt unresponsive (GFP Negative) subpopulations. Sorted cells were exposed to one or both ligands (Wnt3a and TGFB1) and pSMAD3 analyzed by western blot. C. TCF/LEF-GFP reporter expressing Ewing sarcoma cell lines were stably transduced with a Smad binding element- luciferase reporter and then sorted into GFP positive and GFP negative subpopulations as described in (B) prior to treatment with TGFB1. Fold change in luciferase activity (arbitrary units of luminescence) in the subpopulations was measured and results are shown for 3 independent experiments. D. Dual reporter Ewing sarcoma cells containing a TCF/LEF-GFP reporter and SBE-mCherry reporter were pretreated

with Wnt3a prior to treatment with TGFB1 +/- SB505124. FACS plots show percentage of GFP positive (Wnt responsive), mCherry positive (TGF-beta responsive), and double positive (Wnt and TGF-beta responsive) cells in a representative experiment in CHLA9. E. Quantification of mCherry positive cells across three independent replicate experiments for A673, CHLA9, CHLA10, and TC32 as in (D). P-values were determined using student's t-test. *p<0.05, ** p<0.005

These studies show that heterogeneous sub-populations of Ewing sarcoma cells exist *in vitro* and that when Wnt3a and TGFB1 ligands are present in the media, these cells show differential responses with respect to activation of downstream signaling pathways. Specifically, in response to exogenous Wnt and TGF-beta ligands, some cells activate either Wnt/beta-catenin or TGF-beta dependent signaling, some activate both pathways, and others activate neither. This led us to question whether such heterogeneity is also present *in vivo*. To answer this question, we used an orthotopic xenograft model and injected Ewing sarcoma cells directly into the femur of immune deficient mice. Immunohistochemical analysis of adjacent tumor sections identified both pSMAD2 and pSMAD3 positive and negative cells. Additionally, in areas of high pSMAD2 staining, both LEF1+ and LEF1- cells could be identified (**Supplemental Figure 3.1D**). Thus, tumor cell subpopulations with differential Wnt and TGF-beta pathway activation states exist in Ewing sarcoma and canonical Wnt activation primes cells to respond to TGF-beta.

Wnt-sensitized TGF-beta responsive cells upregulate expression of ECM-protein encoding genes

TGF-beta signaling plays a paradoxical role in carcinoma progression wherein it restrains local tumor growth at early disease stages but promotes metastasis at later

stages by activating tumor cell migration and invasion and by inducing pro-metastatic changes in the TME (26). In particular, TGF-beta activated cells induce genes involved in ECM organization (27). We therefore tested whether these previously established TGF-beta targets were impacted by TGF-beta in Ewing sarcoma cells, either alone or following Wnt3a priming, focusing on ECM proteins which we previously identified in the Wnt-dependent secretome (3). As shown, *TNC, COL1A1*, and *CTGF* were all reproducibly induced by TGFB1 and pre-treatment with Wnt3a enhanced this induction, albeit to a small degree **(Figure 3.4A**). In addition, exposure of dual-ligand treated cells to SB505124 blocked induction of the TGF-beta target genes demonstrating that the Wnt-dependent activation of these genes is mediated by TGF-beta, downstream of Wnt/beta-catenin (**Figure 3.4C**).

The observation that induction of previously identified Wnt/beta-catenin targets in Ewing sarcoma is mediated by TGF-beta signaling led us to question whether other elements of the canonical Wnt-dependent transcriptome are also induced indirectly and downstream of TGF-beta. In support of this possibility, we noted that TGF-beta ligand secretion increased in the Wnt-dependent Ewing sarcoma secretome (3), raising the possibility that autocrine TGF-beta signaling may be induced in Wnt-responsive tumor cells even in the absence of exogenous TGF-beta ligands. To test this hypothesis, we performed RNA sequencing analysis of vehicle and Wnt3a treated cells TC32 cells, with and without SB505124 (**Figure 3.4D**). Wnt3a led to significant upregulation of 144 transcripts and induction of 77 of these targets was inhibited by SB505124, demonstrating that Wnt3a-dependent upregulation was mediated by activation of TGF-beta signaling (**Supplemental Table 3.1**). Gene ontology analysis of these 77

Wnt/TGF-beta dependent targets using the DAVID database (20) revealed striking enrichment for ECM organization and TGF-beta signaling, validating our hypothesis that many Wnt3a-induced ECM target genes are, in fact, induced by TGF-beta signaling (**Figure 3.4E**). Notably, among these TGF-beta dependent Wnt targets were the ECM proteins *TNC, COL1A1, MATN3, COL1A2, COL6A3*, and *COL3A1*, all of whom were previously identified in the Wnt dependent secretome (3). Additionally, proteins involved in ECM degradation and remodeling such as member of the disintegrin and metalloprotease (ADAM) family, *ADAM19*, along with matrix metalloprotease 15 (*MMP15*) were also inhibited with addition of the TGF-beta inhibitor. Many of these proteins have been previously shown to be upregulated by TGFB1 in other cancers, however this is the first study in Ewing sarcoma showing that their expression is regulated by the TGF-beta pathway (27). These findings together show that activation of the canonical Wnt-dependent transcriptome leads to changes in expression of ECM genes that are critical to composition of the local TME but that Wnt-mediated activation of these genes is mediated indirectly, *via* TGF-beta.

Wnt activation is correlated with activation of the AngioMatrix

TGF-beta signaling plays a central role in multiple aspects of the TME, impacting on tumor cells, non-tumor stromal, cells, and the ECM. Given our finding that ECM genes are specifically induced by Wnt/TGF-beta-stimulated Ewing sarcoma cells, and that Wnt-stimulated cells secrete ECM proteins, we next sought to determine how these changes might functionally impact on tumor biology. Importantly, TNC, COL1A1, and CTGF all contribute to angiogenesis and their transcripts are members of the

AngioMatrix, a set of 110 ECM and ECM associated genes that are upregulated during the angiogenic switch (28). Therefore, we hypothesized that Wnt/TGF-beta activation in Ewing sarcoma cells might promote an angiogenic phenotype in the adjacent TME. To address this hypothesis, we returned to GSEA analysis of *LEF1*-correlated genes in the two tumor cohorts (GSE63157 and GSE34620) and discovered that angiogenesis (**Figure 3.5A**) and AngioMatrix (**Figure 3.5B**) gene sets highly and reproducibly correlated with *LEF1*. We then interrogated our previous RNA-sequencing study of canonical Wnt-activated Ewing sarcoma cells (5) (GSE75859) and found that 17 of 110 AngioMatrix genes were upregulated downstream of Wnt activation (p=0.019) (**Table 3.2**). Moreover, AngioMatrix proteins were also highly enriched in the Wnt-activated Ewing sarcoma cell secretome (3) (10/39 proteins; p=3.46e-4) (**Table 3.2**). Thus, AngioMatrix genes and proteins are induced downstream of Wnt/beta-catenin activation in Ewing sarcoma. Next, we calculated the composite AngioMatrix score for all tumors in the two patient cohorts (28), and found direct correlations with expression of *PECAM1*, a cell surface marker for endothelial cells (29) (Pearson correlation r = 0.75 (GSE63157) and r = 0.77 (GSE34620); **Supplemental Figure 3.2**). In addition, AngioMatrix scores correlated directly with *LEF1* (**Figure 3.5E**) and with *TGFBR2* (**Figure 3.5F**). Thus, these genomic and proteomic studies of Ewing sarcoma cell lines and patient tumors support the hypothesis Wnt/TGF-beta activated sarcoma cells induce upregulated expression of the AngioMatrix.

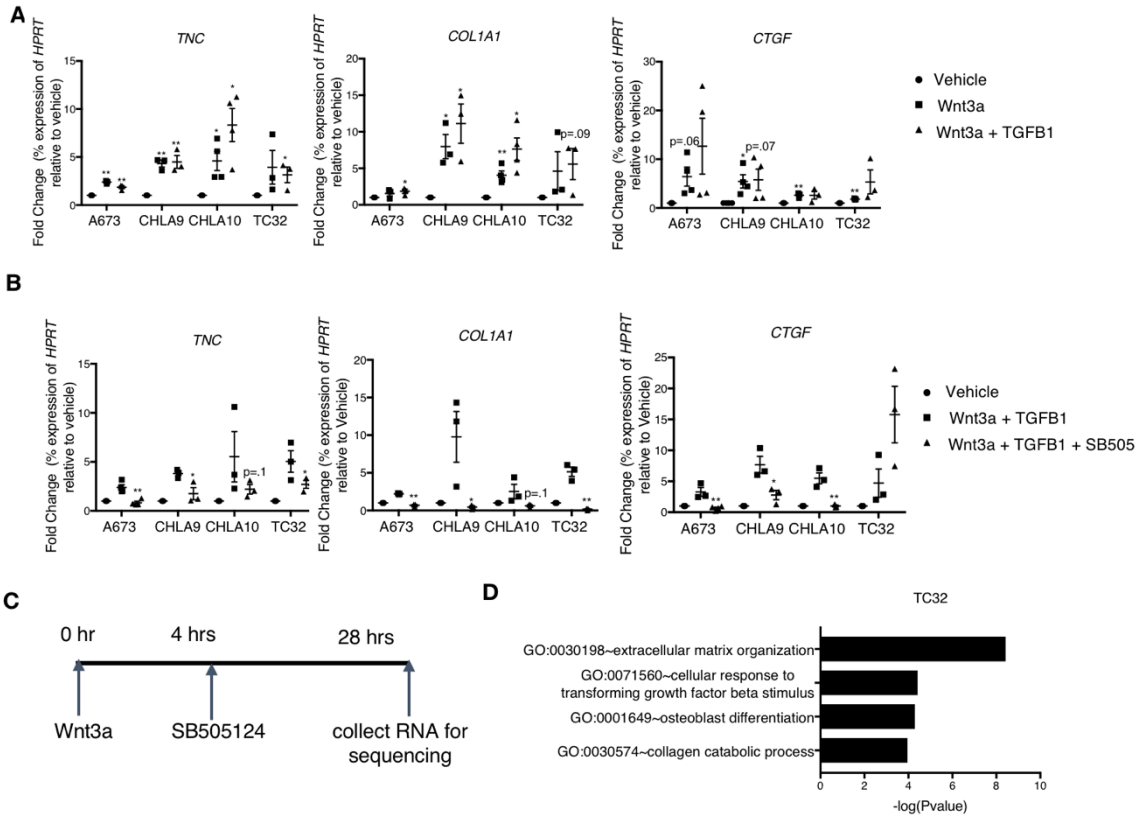

Figure 3.4. Wnt and TGF-beta responsive cells upregulate expression of ECM-protein encoding genes. A. Ewing sarcoma cells treated with both Wnt and TGFB1 induce expression of *TNC*, *COL1A1*, and *CTGF* - all TGF-beta targets important in the ECM. B. Ewing sarcoma cells treated with Wnt and TGF-beta were treated with SB505124 (SB505 in figures), a TGF-beta inhibitor, which blocks induction of *TNC*, *COL1A1*, and *CTGF*. D. Cells were treated with vehicle or Wnt3a for 4 hours prior to treating with vehicle (DMSO) or SB505124 for 24 hours. Cells were then collected in triplicate and RNA was sequenced. E. Gene ontology of transcripts upregulated with Wnt3a and blocked by SB505124 was determined using DAVID. Top 4 gene ontology terms for biological process are shown. All qRT-PCR shown were performed in triplicate and p-values were determined using student's t-test. *p-value <0.05 **p-value <0.005

Transcriptome	Secretome
TIMP1	LUM
MMP2	FMOD
SERPINF1	BGN
SERPINH1	TGFB1
SULF2	COL1A1
ANXA2	COL1A2
COLEC12	COL3A1
CCL2	TNC
COL6A1	CTGF
FN1	TGFBI
TNC	
SRPX2	
MGP	
MFAP4	
SPARC	
AEBP1	
IGFBP5	

Table 3.2. List of Wnt activated transcripts (CHLA25, *GSE75859*) also found in the angiomatrix gene set (p=1.87e-2). List of Wnt induced secreted proteins (TC32 and CHLA10, *PXD007909*) also found in the angiomatrix gene set (p=3.46e-4). For all overlaps chi-squared goodness of fit test was used to determine statistical significance.

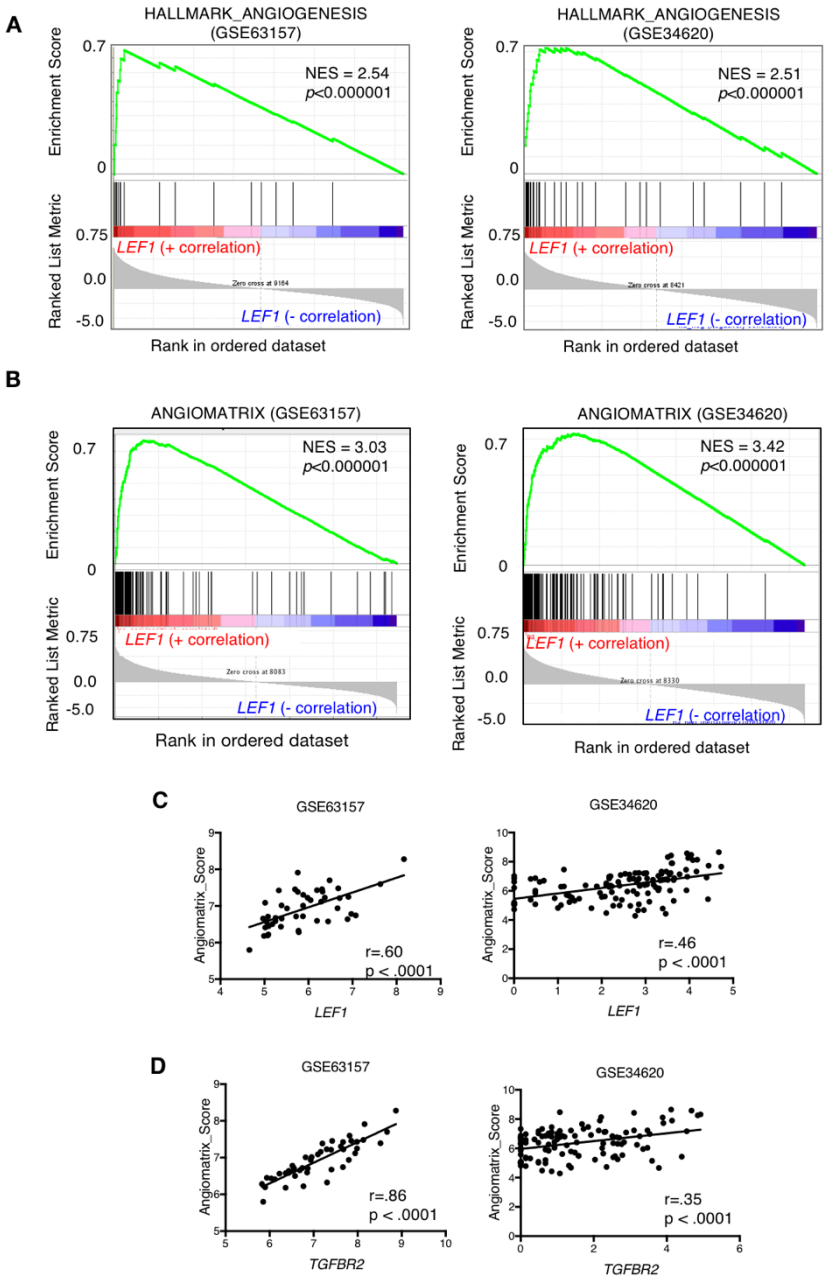

Figure 3.5. Wnt activation is correlated with activation of the AngioMatrix. A. GSEA of whole transcriptome data from independent cohorts of N=46 (GSE63157) and N=117 (GSE34620) patient tumors reveals transcripts that are highly correlated with *LEF1* are enriched with an angiogenesis gene signature. B. GSEA of the same transcriptome data as in (A) in two independent patient cohorts (N=46 and N=117) shows that transcripts highly correlated with *LEF1* expression are enriched for the AngioMatrix gene set. AngioMatrix score was calculated by averaging log2(signal intensity) of all AngioMatrix genes in two independent patient datasets (N=46 and N=117). Correlation of the AngioMatrix score with *LEF1* is shown in (C) and correlation with *TGFBR2* is shown in (D). *LEF1* and *TGFBR2* levels are expressed as log2(signal

intensity). All correlation coefficients shown are Pearson r values and p-values are determined using a student's t-test.

Beta-catenin activated Ewing sarcoma cells promote angiogenesis in the local TME

Given the reproducible association between *LEF1* expression and the AngioMatrix signature, we next sought to determine whether Wnt/bet-catenin activation directly impacts on tumor angiogenesis. To address this, we used Ewing sarcoma cells engineered to stably express constitutively active beta-catenin, as previously described (EBP cells; (5)), after first confirming that they express high levels of *LEF1*, *AXIN2*, *TGFBR2*, and *TNC* compared to empty vector controls (**Figure 3.6A**). To directly evaluate angiogenesis in the TME, we took advantage of the chick chorioallantoic membrane (CAM) assay, which is a well-established model of tumor-induced angiogenesis that allows study of tumor: TME interactions in the context of a physiologic microenvironment (30, 31). Both control and EBP-transduced Ewing sarcoma cells readily formed tumors on the CAM (**Figure 3.6B & D**). Significantly, a reproducible increase in blood vessel formation was observed in the context of EBP tumors (**Figure 3.6C & E**), indicating that beta-catenin-activated Ewing sarcoma cells promote angiogenesis in the context of a physiologic microenvironment.

We next sought to determine the mechanism through which activation of beta-catenin contributes to an angiogenic phenotype. Having established that activation of canonical Wnt signaling alters the Ewing sarcoma secretome, specifically leading to increased secretion of AngioMatrix proteins, we hypothesized that secreted factors from beta-catenin-activated cells contribute to the angiogenic phenotype. To address this

hypothesis, we added conditioned media (CM) from control and EBP A673 and CHLA10 cells to human umbilical vascular endothelial cells (HUVEC). We found that EBP CM increased HUVEC proliferation (**Figure 3.7A**) and viability (**Figure 3.7B**) compared to control CM. The effect was observable as early as 24 hours after addition of the CM but was most significant after 5 days in culture. Notably, there was no differential effect of EBP on endothelial branching morphogenesis in response to CM from beta-catenin activated Ewing sarcoma cells (**Supplemental Figure 3.3**). Thus, secreted factors in the CM of tumor cells with activated beta-catenin promote endothelial proliferation and survival.

As shown in **Table 3.2** the Wnt activated secretome is comprised of AngioMatrix proteins, including TNC. TNC is a known mediator of pro-angiogenic phenotypes and has been shown to be critical in driving the angiogenic switch (32-35). Additionally, TNC has been identified to specifically activate proliferation of HUVECs through binding to integrin alpha-9 (36). Therefore, we tested whether TNC contributes to the observed angiogenesis phenotype, specifically the increase in HUVEC proliferation and viability. We first verified that TNC is induced and secreted by EBP cells compared to controls (**Figure 3.7C**). We then used a monoclonal antibody against TNC to neutralize the secreted protein in CM. As shown in **Figure 3.7D**, neutralization of secreted TNC inhibited the induction of HUVEC proliferation in response to EBP CM, and also abrogated their enhanced viability (**Figure 3.7E**). Taken together, these studies implicate tumor cell-secreted TNC as a key mediator of canonical Wnt-induced angiogenesis in the Ewing sarcoma TME.

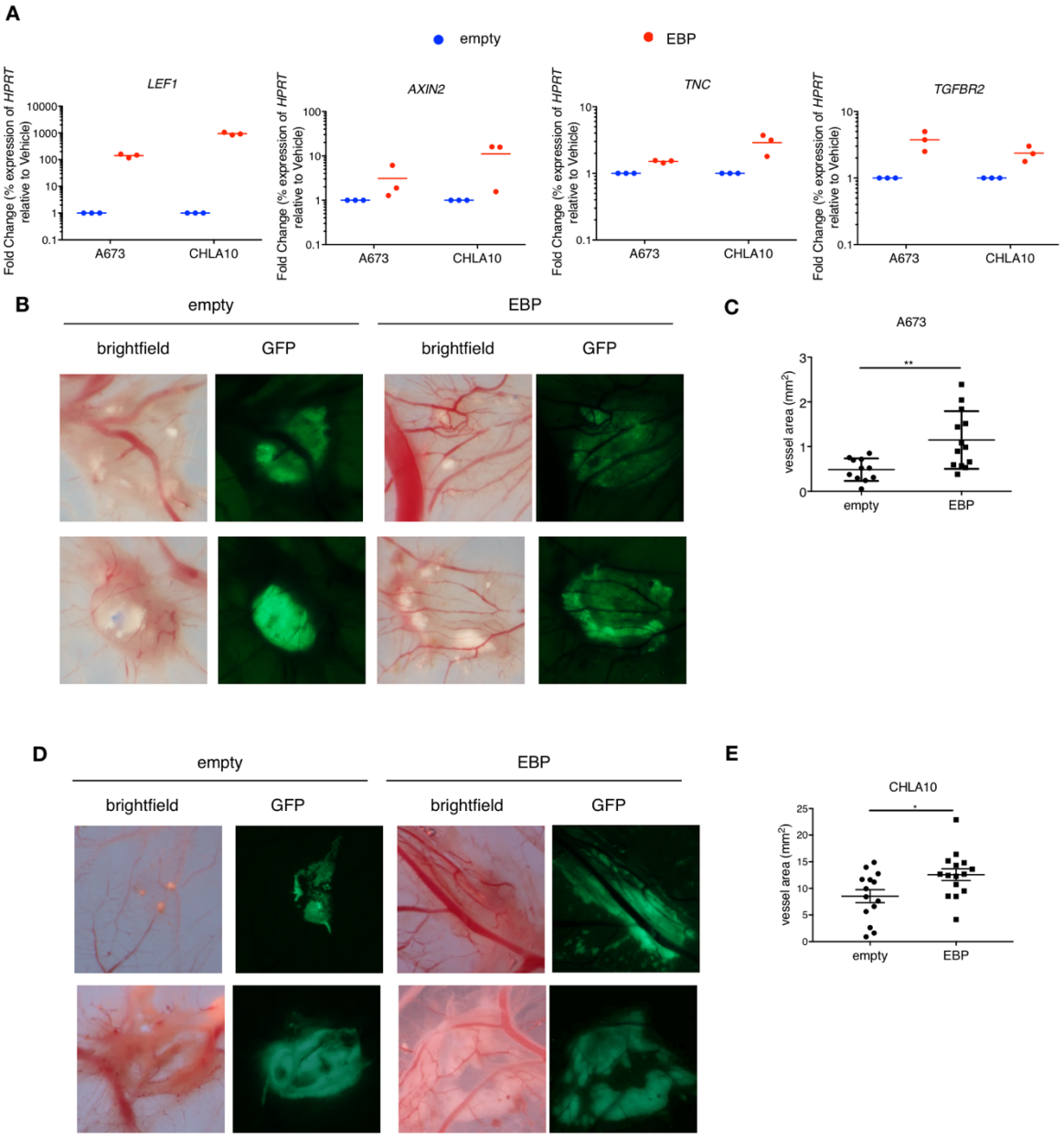

Figure 3.6. Beta-catenin activated Ewing sarcoma cells show increased angiogenesis. A. Ewing sarcoma cells engineered to express constitutively active beta catenin (EBP) demonstrate reproducible upregulation of canonical targets *LEF1, AXIN2, TGFBR2, and TNC* in three independent replicates. Tumor cells were placed on the chick chorioallantoic membrane (CAM) for 72 hours. Tumors were located by GFP, and blood vessels were photographed under a stereoscope. In both A673 (B) and CHLA10 (D), EBP tumors had a significantly higher area of blood vessels compared to empty vector control tumors. Quantification of blood vessel area is shown for A673 (C) and CHLA10 (E). *p<0.05, **p<0.01, n ≥ 12 independent experiments per condition.

Wnt/beta-catenin, TGF-beta, and the AngioMatrix are correlated with poor prognosis in patient cohorts

Prior studies of other tumors have demonstrated that a high AngioMatrix score is associated with worse clinical outcomes ((28)). Therefore, we sought to determine if a relationship exists between the AngioMatrix, Wnt-beta catenin, TGF-beta, and survival in Ewing sarcoma tumors *in vivo*. Consistent with other cancer types, Ewing sarcoma tumors with high AngioMatrix scores had higher rates of patient relapse and death than tumors with low scores (**Figure 3.8A**). We previously reported that high *LEF1* expression was associated with a worse prognosis in these patients (5) and high *TGFBR2* was, likewise, associated with diminished survival (**Figure 3.8B).** Importantly, a 33-gene prognostic gene signature was previously reported for this patient cohort and was found to be inextricably linked to the presence of stroma, in particular to the presence of abundant fibrovascular matrix in tumor biopsies (6). *LEF1* expression in these tumors was also found to correlate directly with poor prognosis genes, and inversely with good prognosis genes, in the 33-gene signature (**Figure 3.8C**). These *LEF1* correlations were found to be reproducible and even more significant in an independent patient cohort (**Supplemental Figure 3.4A**). Notably, many of the most significantly correlated genes in both datasets are specifically involved in angiogenesis (e.g. *ENG*, *HEY2, IL6, ITGA9, VEGFC*, and *VWF*). We therefore hypothesized that beta-catenin activation might serve as a key regulator of stromal deposition in Ewing sarcoma. In support of this, higher expression of *LEF1* was detected in stroma-rich than stroma-poor tumors (**Figure 3.8D**) and, as predicted, tumors with abundant stroma also had higher AngioMatrix scores (**Figure 3.8E**)**.** In keeping with their induction

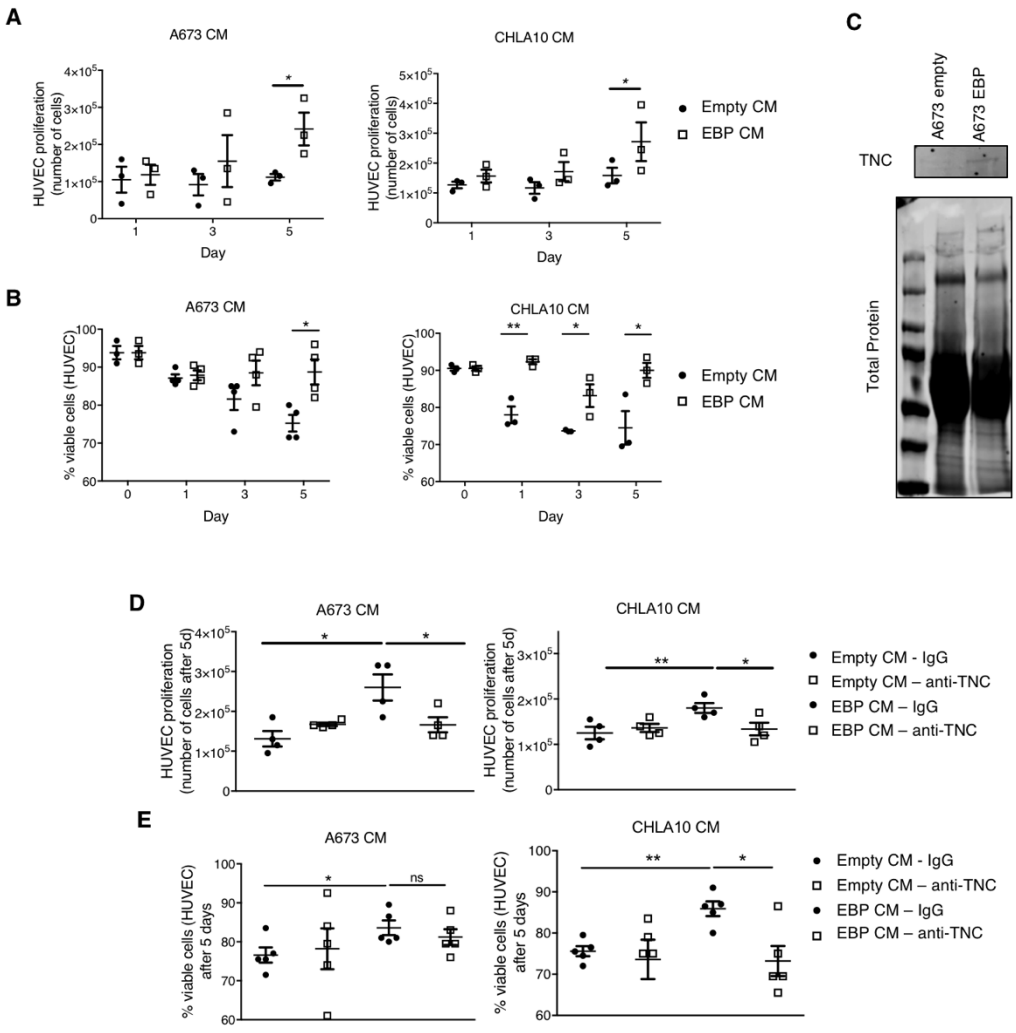

Figure 3.7. Beta-catenin activated Ewing cells secrete factors that promote HUVEC proliferation. Conditioned media (CM) from A673 and CHLA10 cells containing either empty or EBP vector was collected and added to HUVECs. Proliferation (A) and viability (B) of HUVECs was measured at day 1, 3, and 5 post addition of Ewing's CM. A statistically significant increase in both proliferation and viability of HUVECs was observed after 5 days of CM from EBP cells as compared to empty vector CM. C. TNC secretion is increased in Ewing's cells with the EBP vector in both A673 and CHLA10. In both CHLA10 and A673 cells, inhibition of TNC with a monoclonal antibody (anti-TNC) but not with control antibody (IgG) abrogates the effect of EBP CM on proliferation (D) and viability (E) of endothelial cells. *p<0.05, **p<0.01, n ≥ 3 independent experiments.

downstream of Wnt/beta-catenin activation, *TGFBR2, TNC,* and *COL1A1* were also more highly expressed in stroma-rich biopsies (**Figure 3.8F**). The absence of

differences in expression of canonical Wnt targets that are not induced by beta-catenin in Ewing sarcoma cells (e.g. *MYC, CCND1, LGR5*; **Supplemental Figure 3.4B**) supports the conclusion that Wnt-activated tumor cells, rather than stromal cells, contributed to higher expression of *LEF1* in stroma-rich biopsies.

Thus, the gene expression profiling studies of primary tumor biopsies reveal that highly significant and reproducible correlations exist in Ewing sarcoma between Wnt/beta-catenin signaling, *TGFBR2* expression, and the AngioMatrix, corroborating our findings from experimental models. In addition, cell line models and patient tumor biopsies confirm that there is substantial inter- and intra-tumor heterogeneity with respect to Wnt/beta-catenin and TGF-beta signaling. Altogether, our results support the conclusion that functional cooperation between canonical Wnt and TGF-beta plays a key role in mediating tumor angiogenesis and disease progression and that this cooperation is dependent on dynamic crosstalk between subpopulations of Ewing sarcoma tumors cells and their TME.

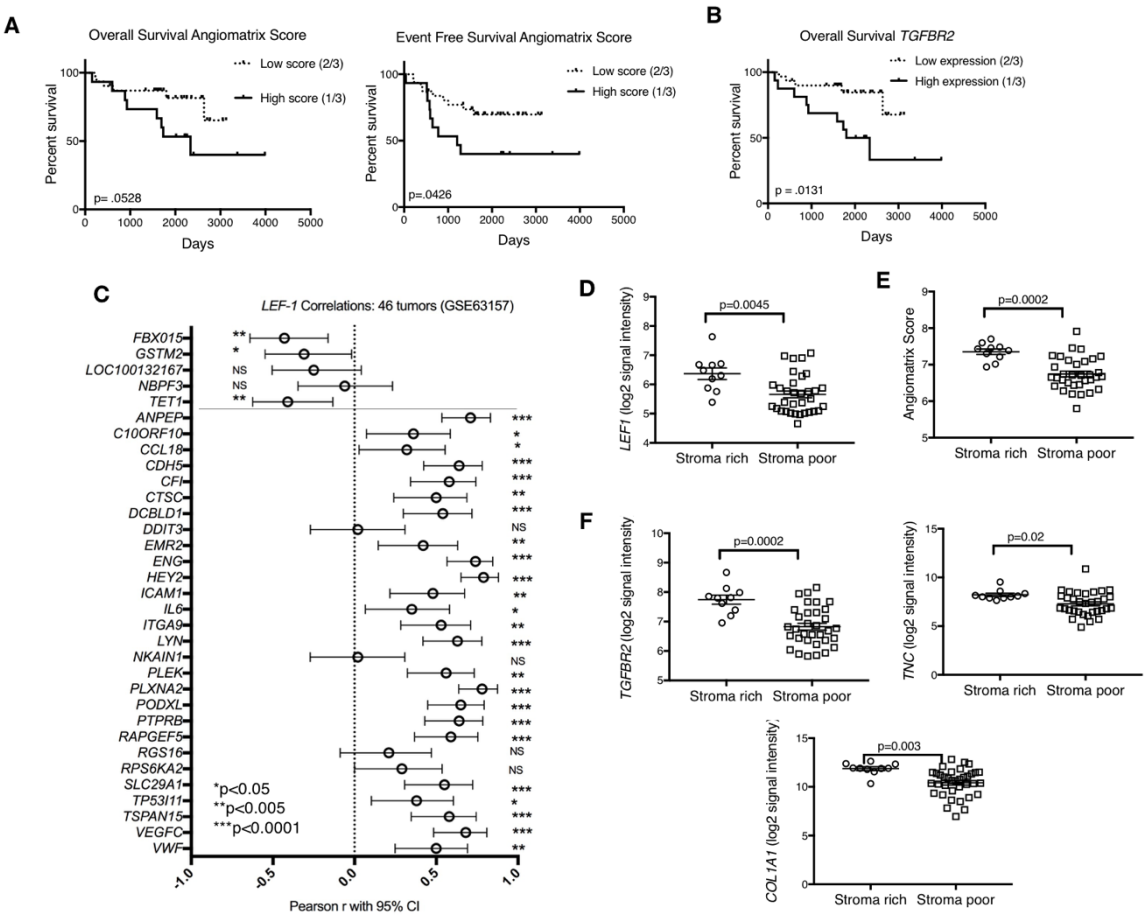

Figure 3.8. Wnt/beta-catenin, TGF-beta, and the AngioMatrix are correlated with poor prognosis in patient cohorts. A. High AngioMatrix scores are associated with worse event-free and overall survival in patients with localized disease (GSE63157). B. High TGFBR2 expression is associated with worse overall survival. C. 43 patient tumors were grouped based on stroma content (stroma rich < 70% tumor content (N=10); stroma poor > 70% tumor content (N=33)). *LEF1* expression and D. AngioMatrix scores in stroma rich tumors vs. stroma poor tumors. E. *TNC, TGFBR2,* and *COL1A1* expression in stroma rich and stroma poor tumors. All p-values were determined using student's t-test. F. Pearson correlations (r) of *LEF1* expression (error bars: 95% confidence intervals (CI)) with 33 gene prognostic signature. Top 5 genes were identified as good prognosis biomarkers in this patient cohort. High expression of bottom 28 genes was associated with poor prognosis. NS indicates not significant.

Discussion

In this work, we provide evidence that activation of Wnt/beta-catenin signaling changes tumor: TME crosstalk to promote Ewing sarcoma disease progression by

cooperating with TGF-beta to increase expression of the AngioMatrix, and promote angiogenesis. Previous studies using subcutaneous xenografts and orthotopic xenografts in the tibia showed an increased propensity of metastasis when cells were in the bone TME (37). Additionally, identification of a poor prognostic gene signature in a patient cohort containing tumors with high stromal content, also indicated a role for the TME in driving more aggressive tumors (6). Here, we have built on these previous findings and specifically show a role for Wnt/beta-catenin signaling in dictating tumor: TME changes and suggest that a TME rich in Wnt ligands (i.e. a bone TME), would promote transition to a metastatic phenotype through secretion of the AngioMatrix and activation of angiogenesis.

Previous studies from our group have indicated a role for Wnt/beta-catenin activation in EWS-FLI1 antagonism in Ewing sarcoma (5), resulting in derepression of EWS-FLI1 targets in Wnt activated cells. We have reported that activation of Wnt/beta-catenin leads to derepression of the EWS-FLI1 target, *TGFBR2*, contributing to cell plasticity by transitioning cells from a TGF-beta unresponsive to TGF-beta responsive state. We also identified subpopulations of cells that are Wnt and TGF-beta responsive, Wnt responsive, TGF-beta responsive, or responsive to neither using our dual reporter system, further supporting findings from our group and others that Ewing's tumors are heterogenous (3, 5, 8, 38-40). Among this heterogeneity is the observation that EWS-FLI1 levels are variable between different cells, and that a population of EWS-FLI1 low cells exist in Ewing sarcoma tumors (39). We have hypothesized that this small subpopulation of TGF-beta responsive cells are indeed the EWS-FLI1 low cells, and by priming with Wnt3a, we are antagonizing EWS-FLI1 resulting in a larger population of

cells that are EWS-FLI1 low and subsequently TGF-beta responsive. However, the mechanism through which Wnt antagonizes EWS-FLI1 remains largely unknown and is outside the scope of this manuscript.

Induction of angiogenesis is a hallmark of cancer (41). Ewing sarcoma tumors, like all cancers, require a vascular supply to maintain growth and disease progression. Major known regulators of angiogenesis in Ewing sarcoma include vascular endothelial growth factor (VEGF) (42-46) and insulin like growth-factor (IGF) signaling (47-49). Wnt signaling contributes to angiogenesis in numerous contexts. Under physiologic conditions, Wnt/beta-catenin activation and *LEF1* expression in endothelial cells promote pro-angiogegnic phenotypes including proliferation and migration of endothelial cells (50-52). Furthermore, effects of Wnt signaling on endothelial pathogenesis and promotion of angiogenesis has been described in numerous disease states including cancer (52, 53). In cancer, Wnt signaling can promote angiogenesis by induction of secreted factors such as VEGFs, matrix metalloproteinases (MMPs), and basic fibroblast growth factor (bFGF) that promote pro-angiogenic phenotypes in endothelial cells (9, 54). Similarly, multiple cancers have demonstrated downregulation of the Wnt-inhibitory molecule DKK1 during tumor progression, which is associated with increased VEGF expression and vascular density (55, 56). We are able to explore how Wnt/beta catenin activation in the tumor cells themselves is able to contribute to angiogenesis. Our data reveals that *LEF1* expression is highly correlated with poor prognostic genes in two independent tumor cohorts. Notable genes in this dataset include VEGF family members and genes involved in the vascular endothelial growth factor receptor signaling pathway (**Figure 3.8 and Table 3.1**). VEGF family members, in particular the

VEGF165 isoform, are major mediators of angiogenesis in Ewing sarcoma (46, 57). We have also found that Wnt/beta-catenin promotes angiogenic phenotypes *in vivo* and in the chick CAM assay, and these phenotypes are in part mediated by tumor cell specific secreted factors that promote endothelial proliferation and survival.

TGF-beta activation is supportive of both tumor suppressing and tumor activating processes, depending on the tumor context in carcinomas (22). Where as previous studies supported a role for TGF-beta to be tumor suppressive in early stages of Ewing sarcoma (24), we hypothesize that activation of TGF-beta as the tumor becomes more metastatic (i.e. through Wnt activation) plays a tumor promoting rather than tumor suppressive role in sarcomas. Specifically, TGF-beta signaling has been shown to promote angiogenesis, invasion, and metastasis through induction of EMT (22, 58). Interestingly, we show here that genes with high *LEF1* correlation in patients are enriched for the EMT signaling pathway. Although Ewing sarcoma cells are initially mesenchymal, increasing evidence in sarcomas supports a role for an EMT like change resulting in increased cancer invasiveness (23). In particular, in Ewing sarcoma higher levels of *ZEB2*, a transcription factor upregulated in EMT, contributes to a more mesenchymal like and invasive phenotype, while lower *ZEB2* levels better resemble epithelial like states (40). Activation of Wnt/beta-catenin had also previously been shown to induce transition to a more metastatic cell state through increased migration, an EMT associated process. Based on this data and the data we present here, we hypothesize that Wnt activated cells are are more responsive to TGF-beta, and activation of the TGF-beta pathway in Wnt primed cells induces an EMT like switch, including promotion of angiogenesis, migration, and invasion.

Using a RIP1-Tag2 model to model the angiogenic switch, a group of ECM associated proteins categorized as the AngioMatrix, and specifically TNC, were shown to be critical in driving the angiogenic switch (28). Interestingly, many of those genes found in the AngioMatrix are also induced by Wnt/beta-catenin in Ewing sarcoma cells and are targets of the TGF-beta pathway. In particular we show that *TNC*, *COL1A1*, and *CTGF*, all members of the AngioMatrix, are mediated by TGF-beta dependent signaling. Additionally, we saw a correlation of the AngioMatrix score with *LEF1* and *TGFBR2* in two independent patient cohorts, showing a link between Wnt/beta-catenin, TGF-beta response, and the AngioMatrix. Both TGF-beta and Wnt/beta-catenin play a large role in angiogenesis by directly binding to endothelial cells to regulate endothelial cell proliferation and vascular formation through activation of AngioMatrix secretion (51, 54, 59). However, little is understood about the tumor specific contribution to the AngioMatrix. Here, we show that activation of both Wnt and TGF-beta in Ewing sarcoma results in tumor cell specific expression and secretion of the AngioMatrix, implying that tumor cells themselves can be critical in mediating the angiogenic switch.

Finally, we show that induction and secretion of the AngioMatrix protein TNC is necessary for these Wnt/beta-catenin activated cells to promote HUVEC proliferation and the angiogenic phenotype. TNC is a multi-functional extracellular matrix protein that is both membrane bound and secreted, and is known to contribute to angiogenesis in normal and pathologic settings, in particular through promotion of endothelial proliferation and survival (32, 33, 60). While most studies show promotion of proliferation by TNC, in some experimental systems TNC antagonizes proliferation (34), demonstrating the context-specific function of this molecule. TNC signals through alpha-

v-beta-3 and alpha-9-beta-1 integrins to activate ERK and FAK signaling which promotes cellular proliferation, migration, and survival through inhibition of apoptosis (61-63). Given the strong association between *LEF1* and *ITGA9* (which encodes the alpha subunit of alpha-9-beta-1 integrin) in primary tumors (**Figure 3.8**), as well as enrichment for integrin and ERK signaling (**Table 3.1**), it is likely that the endothelial phenotypes observed in response to EBP conditioned media are through TNC signaling via these integrin receptors to promote endothelial proliferation and survival. In addition to functioning as a direct ligand to induce signal activation of pro-angiogenic phenotypes, TNC may also promote angiogenesis through other mechanisms. In glioma, TNC is associated with poor outcomes, and promotes angiogenesis indirectly through expression of the cell surface receptor ephrin B and its associated downstream signaling pathways (34). In breast cancer, TNC binds to multiple proteins and may function to bring protein moeities into closer proximity and thus modify cellular signaling (18). Of particular interest, VEGF, as well as other pro-angiogenic molecules such as PDGF, IGF binding proteins, TGF-beta, and FGF (56, 64) have been demonstrated to bind to TNC. The ability of TNC to bind to multiple pro-angiogenic proteins and modify their physical proximity raises the intriguing possibility that *TNC* expression may augment and impact the effects of a pro-angiogenic milieu in the extracellular microenvironment.

Taken together, these data demonstrate a novel mechanism by which Wnt/beta-catenin signaling, TGF-beta signaling, and *TNC* promote aggressive disease, namely through promotion of angiogenesis and alterations of tumor: TME crosstalk. We see that ligands in the TME can signal to tumor cells resulting in heterogeneous cell state

transitions. Cells can then signal back to the TME through secretion of proteins, suggesting that a bidirectional relationship exists between the TME and Ewing sarcoma cells. We specifically note the presence of heterogeneous subpopulations of Wnt and/or TGF-beta responsive cells, highlighting the presence of tumor heterogeneity and plasticity in Ewing sarcoma. Different subpopulations of tumor cells are able to communicate differently with the local TME, altering local tumor: TME crosstalk to promote processes such as angiogenesis, which could be a key regulator of Ewing sarcoma metastasis. Therapeutic strategies should focus on targeting the TME and secreted contents of tumor cells in order to eliminate signals between the tumor: TME that drive Ewing sarcoma progression. Specifically, targeting TNC could inhibit TNC-mediated tumor:TME interactions, such as angiogenesis. The data presented here provides rationale for further investigation to the implications of tumor: TME crosstalk and how to better target these interactions to inhibit tumor metastasis.

Methods

Cell culture and reagents

Ewing sarcoma cell lines were obtained from the COG cell bank (cogcell.org), and identities of the cells were verified by short tandem repeat (STR) profiling. Cells were routinely tested for mycoplasma contamination and verified to be negative. A673 and TC32 cells were cultured in RPMI 1640 media (Life Technologies, Waltham, MA) supplemented with 10% fetal bovine serum (FBS)(Atlas Biologicals, Fort Worth, CO) and 2mM L-glutamine (Life Technologies). CHLA9 and CHLA10 cells were culture in IMDM media (Life Technologies), 20% FBS, 2 mM L-glutamine, and 1X insulin-transferrin-selenium (ITS) supplement. Human umbilical vascular endothelial cells

(HUVEC) were purchased from Lonza and cultured in medium 200 with low serum growth supplement (LSGS) (Thermo Fisher, Waltham MA). Cells were maintained at a low passage for all experiments. GFP-tagged cells expressing a constitutively active beta-catenin construct (EBP) or corresponding empty vector control were generated as previously described (5). To activate canonical Wnt signaling, cells were treated with recombinant human Wnt3a (R&D, 5036-WN) at a concentration of 100 ng/mL. To activate TGF-beta signaling, cells were treated with recombinant human TGF-beta 1 (TGFB1) (R&D, 240-B) at a concentration of 10 ng/mL. To inhibit the TGF-beta signaling pathway, cells were treated with the ALK inhibitor SB-505124 (Cayman Chemical, 694433-59-5) at a concentration of 10 uM.

Clinical correlations and gene set enrichment analyses

Tumor gene expression data were extracted from previously published independent datasets from the Children's Oncology Group (COG) (GSE 63157 (6)) and from European collaborative studies (GSE 34620 (19)). Correlations with *LEF1* were measured by Pearson correlation and 95% confidence intervals determined. An *in vivo* signature of Wnt/beta-catenin signaling was generated by ranking genes based on correlation with *LEF1* expression in the aforementioned data sets, and gene set enrichment analysis (GSEA) was performed using the GseaPreranked function of GSEA v2.1.0 software (Broad Institute) (21). Gene ontology enrichment was determined using the same list of genes with a cutoff of Pearson r>0.5, and interrogated using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (20).

Western Blotting

For phospho-SMAD3 (pSMAD3) western blots, cells were pretreated with vehicle or Wnt3a (100 ng/mL) for 24 hours prior to TGFB1 (10 ng/mL) for 15 minutes. Whole cell lysates were isolated using RIPA buffer + phosphatase inhibitor tablet (Roche, Cat # 4906837001) + protease inhibitor tablet (Sigma, Cat # 4693116001). For secreted protein, CM from EBP and control cells was concentrated using three kilodalton cutoff Amicon Ultra Centrifugal Filter Units (Fisher, UFC900308). Protein concentration was measured using the DC Protein Assay (Bio-Rad). Western blot analysis was performed using the Bio-Rad Mini-PROTEAN Tetra System. Following transfer, nitrocellulose membranes were blocked in 5% BSA in TBS-T for 1 hour. Membranes were washed once with TBS-T and then incubated overnight at 4^{o} C with either mouse-anti human Tenascin C (1:500, Sigma T2551) or rabbit anti-phospho-Smad3 (1:500, Cell Signaling 9520). Membranes were washed three times in 1X TBS-T for 5 minutes each and then incubated with secondary antibody IRDye 800CW goat anti-mouse (1:10,000, LiCor) or IRDye 800CW goat anti-rabbit (1:10,000, LiCor) for 1 hour. They were then washed two times with 1X TBS-T and once with 1X TBS for 5 minutes prior to scanning the membrane using the LiCor Imaging system. Western blot analysis of secreted TNC was performed as previously described in (3).

Reporter Studies and Flow Cytometry

Ewing sarcoma cells stably transduced with a TCF/LEF-GFP reporter as previously described in (5), were transduced with a lentiviral smad binding element (SBE) – Luciferin reporter (Cignal Lenti SMAD Reporter (luc) Kit: CLS-017L). TCF/LEF-GFP

reporter cells were also transduced with a SBE-mCherry reporter construct, a kind gift of the Elaine Fuchs' lab (25). Cells containing the TCF/LEF-GFP reporter and SBE-luciferin reporter were treated with Wnt3a for 48 hours prior to sorting based on GFP. Cells were plated into a 96 well plate and treated with TGFB1 for 24 hours prior to measuring luciferin using the Pierce Firefly Luciferase Glow Assay Kit (Thermo Fisher, 16176). For all other sorting experiments, cells were treated with Wnt3a for 48 hours before sorting based on GFP positivity and then used for subsequent experiments. For flow cytometry analysis, TCF/LEF-GFP reporter and SBE-mCherry reporter cells were treated with Wnt3a for 24 hours prior to treatment with TGFB1 for 72 hours before flow cytometry analysis for both GFP and mCherry positive cells.

RNA Sequencing Analysis and Gene Expression Analysis

TC32 cells were treated with vehicle (PBS) or Wnt3a (100 ng/mL) for 4 hours prior to treatment with DMSO or SB505124 (10 uM) for 24 hours. RNA was collected for each condition in triplicate and single-end sequencing was done on the Illumina HiSeq 4000 (University of Michigan Sequencing Core). Fastq generation was performed using Illumina's CASAVA-1.8.2 software, analyzed for quality control using MultiQC, and aligned using Star. For differential transcript expression analysis we used the R package, DESeq2. Comparisons were made between vehicle and Wnt+DMSO and then Wnt+DMSO and Wnt+SB505. Genes that were upregulated in Wnt + DMSO vs. vehicle had an adjusted p-value of >.1 and log(fold change) > .6. Genes downregulated in Wnt + DMSO vs. Wnt + SB505 had an adjusted p-value of >.1 and log(fold change) < -.6. Genes that were identified to be differentially expressed in both comparisons along with the complete list of genes that are both up in vehicle vs. Wnt and down in Wnt vs.

Wnt+SB505 are included in supplemental table 1. Gene ontology analysis was performed using DAVID (20). Gene expression was measured using standard methods of qRT-PCR. See supplemental Table 2 for primer sequences.

Angiomatrix Score Calculation, Correlations, and Survival Curves

The angiomatrix score was determined by averaging normalized gene expression in both GSE63157 and GSE34620 for 110 genes identified to be in the angiomatrix from (28). Pearson correlation coefficients and 95% confidence intervals were calculated between the angiomatrix score for each patient and *LEF1*, *TGFBR2*, and *PECAM1*. GSEA was also performed using the GseaPreranked function of GSEA with genes from each patient cohort ranked based on *LEF1* correlation mapped against the angiomatrix gene set (21). Angiomatrix scores for each patient in the N=46 (GSE63157) were separated based on tumor content. Stroma rich were tumors with < 70% tumor content (N=10) and stroma poor were tumors with > 70% tumor content (N=33). 3 patients were excluded from this study because no tumor content information was available. Expression of *LEF1, CCND1, MYC, TGFBR2, TNC*, and *COL1A1* was grouped by stromal content and student's t-tests were used to look for statistically significant differences between groups. Kaplan-Meir survival curves were produced by grouping patients into the top 1/3 and bottom 2/3 for angiomatrix score and log2(signal intensity) of *TGFBR2* in GSE63157.

Chick chorioallantoic membrane (CAM) assay

Fertilized eggs were obtained from the Michigan State University Department of Animal

Science Poultry Farm. Immediately upon arrival, eggs were placed in a humidified, rocking incubator (G.Q.F. Manufacturing, Savannah, GA) at 37°C for 11 days (E11). On E11, eggs were assessed for viability of the embryo using a handheld light source (G.Q.F. Manufacturing). Eggs containing non-viable embryos were discarded. The CAM was "dropped" as previously described (30, 52), and $1x10^6$ GFP-labeled A673 or CHLA10 cells were placed on the CAM in 10 µL of 2.5% growth-factor reduced Matrigel (BD Biosciences) in PBS. Cells were incubated for three days without rocking. On E14, the CAM was dissected out and fixed for 1 hr in 4% paraformaldehyde. Tumors on the CAM were identified and imaged by GFP fluorescence and bright field microscopy on an Olympus SZX16 Stereo-Dissecting macroscope and analyzed using NIS-Elements Imaging software. Vessel area was determined by quantifying the area of red pixels within the area of the tumor (per high powered field).

HUVEC Proliferation and viability assays

Conditioned media (CM) from EBP and control Ewing sarcoma cells was generated by plating $5x10^6$ CHLA10 or $10x10^6$ A673 or TC32 cells on ten-centimeter dishes. Once adherent, cells were serum starved and serum-free CM was aspirated after 24 hours and centrifuged at 900 rcf for 5 min to remove cell debris. CM was added in a 50:50 concentration to HUVEC cells plated at a density of $7.5x10^4$ cells per well on six well plates, in M200 containing 0.25X LSGS. At days 1, 3, and 5, cells were dissociated with Accutase (Corning) and stained with trypan blue (Invitrogen). Cell number and viability were determined using a Countess automated cell counter, and confirmed manually on a hemocytometer under a microscope. Each replicate was counted in duplicate and the

average cell number and viability were determined. Tenascin C was inhibited by addition of a neutralizing mouse monoclonal antibody (T2551, Sigma) at a concentration of 10 ng/ml at the time of CM addition to HUVECs. Mouse IgG1 isotype antibody (sc-2025, Santa Cruz Technologies) was used at the same concentration as a control.

Mouse xenograft studies

A673 cells (2 x 10^5) were injected directly into the femur of 9-12 week old NOD-SCID-gamma mice (Charles Rivers, Boston, MA). Femurs were harvested two weeks after injection, fixed in zinc formalin before decalcification for 12 days in 20% EDTA. Decalcified femurs were paraffin embedded and sectioned. Immunohistochemistry was performed on adjacent sections using hematoxylin and eosin and the following antibodies: mouse anti-human Tenascin C (1:4000, Sigma T2551), LEF1 (1:2000, Abcam ab137872), CD31 (1:200, Cell Signaling Technology 77699S), pSMAD2 (1:100, ThermoFisher Scientific #44-244G) and pSMAD3 (1:100, ThermoFisher Scientific #44-246G) and CD99 (1:250, Abcam ab212605).

Statistics

For all gene expression analysis, student's t-tests were performed and p-values were computed. Comparisons were considered statistical significant with $p<0.05$. Throughout figures, *indicates $p<0.05$ and **indicates $p<0.005$ unless indicated as otherwise. Correlation coefficients computed are all pearson r coefficients. Statistical significance of overlaps was determined using chi squared goodness of fit test. All error bars indicated represent mean $\pm$ SEM.

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Chapter 4: Tenascin C and Src cooperate to promote invadopodia formation in Ewing sarcoma

Summary

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 invasion in Ewing sarcoma 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 cell intrinsic and extrinsic TNC can enhance Src activation and invadopodia formation in Ewing sarcoma. We found that microenvironmental stress significantly 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. Importantly, this work highlights the impact of stress-induced *TNC*

expression on enhancing cell invadopodia formation and provides evidence for a feed forward loop between TNC and Src to promote metastatic behavior in Ewing sarcoma.

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 multi-modality therapy (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 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. 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, angiogenesis and metastatic engraftment (4-7) (Chapter 3). 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 that promote cytoskeleton changes, activation of CXCR4, Src and Rac/Cdc42, angiogenesis, and secretion of extra-cellular matrix (ECM) proteins,

such as tenascin C (TNC) and collagen I, that are known to contribute to pro-metastatic tumor niches (4-7). In particular, Ewing sarcoma cells that are exposed to hypoxia and growth factor deprivation rapidly induce the formation of actin-rich protrusions known as invadopodia, which trigger degradation of the surrounding ECM, through activation of Src kinase (4).

Activation of Src kinase is found in many cancers and effects many processes including, but not limited to, adhesion, migration, invasion, and cell morphology (8). Src activity is mediated by phosphorylation of the negative regulatory Tyr530, displacement of SH3 and SH2 domains through ligand binding, and phosphorylation of the positive regulatory Tyr419 (9). Once Src is activated, its primary role is to mediate cell-cell and cell-matrix interactions. This occurs through interaction of Src with plasma membrane-bound molecular partners, such as integrins to mediate cytoskeletal functions, or receptor tyrosine kinases to alter migration and proliferation (8, 10). As a key regulator of signaling pathways aberrantly activated in cancers, Src is a prime candidate for development of therapeutic targets (11). Of note, we have shown that application of the Src inhibitor dasatinib blocks the invasive phenotype in Ewing sarcoma (4).

Invadopodia are actin-rich protrusions that extend into the ventral surface of invasive cells (12). Accumulation of the actin assembly protein, cortactin, and matrix metalloproteases (MMPs), specifically MMP14, are key factors in mediating formation and function of the invadopodia structure (12, 13). Formation of these invadopodia structures results in coordination of cell motility with ECM degradation. In Ewing sarcoma, we have reported that phosphorylation of Src at the Tyr419 activating site is increased with nutrient deprivation and hypoxia resulting in increased invadopodia

formation which can be pharmacologically blocked by the Src-kinase inhibitor dasatinib (4). However, the mechanism through which this increased invadopodia occurs remains to be determined.

A key regulator of the metastatic phenotype in Ewing sarcoma is the matricellular protein, TNC (5). Activation of Wnt/beta-catenin induces transcription and secretion of TNC resulting in increased angiogenesis and lung engraftment in Ewing tumor xenografts (5, 6) (Chapter 3). 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 (14). 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 (15, 16). TNC expression is limited in adult tissues, but can be induced in instances of injury and tissue remodeling (17). 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 (18-20). Deposition of TNC by breast cancer cells is critical to production of a metastatic niche, supporting the importance of tumor cell-derived TNC in promotion of successful metastatic engraftment (19).

Interestingly, TNC has also been reported in other solid tumors to promote transition to a metastatic cell state and enhance Src phosphorylation (21). Given our prior work independently demonstrating the importance of both Src and TNC in promoting Ewing sarcoma cell metastatic behavior, we sought to determine whether Src and TNC cooperate to alter invadopodia formation in Ewing sarcoma. We hypothesized that TNC is a positive regulator of invadopodia formation in Ewing sarcoma through its

ability to activate Src. In the current study, we investigate the impact of TNC and Src on invadopodia formation by determining both the ability of cell intrinsic and extrinsic TNC to activate Src and conversely, the role of Src activation on TNC expression.

Results

Tenascin C reduces Ewing sarcoma cell spreading

Our previous work demonstrated that tumor cell-derived tenascin C (TNC) is necessary for metastatic lung engraftment in Ewing sarcoma (4). TNC secreted into the TME, by either tumor cells or stromal cells, can alter tumor: TME crosstalk through signaling to tumor cells, stromal cells, and components of the ECM. Specifically, TNC can bind and activate integrins, resulting in decreased or increased cell adhesion, dependent on the context (14, 16). Whether or not TNC contributes to adhesive or counter-adhesive effects in Ewing sarcoma has not been described previously. To determine the impact of exogenous TNC on Ewing sarcoma cell adhesion in culture, tissue culture dishes were coated with recombinant TNC or vehicle and adhesion of Ewing sarcoma cells was assessed. As shown (**Figure 4.1A**), a striking difference in adhesion to the tissue culture plate was noted. Whereas tumor cells readily adhered to control dishes and spread to form classic cell monolayers, under TNC conditions Ewing cells did not adhere to the plate and instead favored cell-cell interactions, growing as three-dimensional spheres (**Figure 4.1A**). Therefore, we conclude that presence of TNC in the TME decreases cell spreading and adhesion of Ewing sarcoma cells.

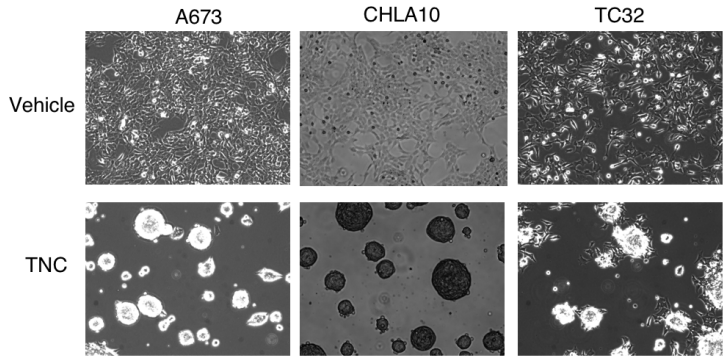

Figure 4.1 Tenascin C reduces Ewing sarcoma cell spreading. Plates were coated with 5 ug/mL recombinant TNC or PBS prior to addition of Ewing sarcoma cells. Pictures of cells were taken 24 hours after seeding. Cells cultured in the presence of TNC are unable to spread onto the plate and form 3D spheroids.

Tenascin C enhances Src phosphorylation

The ability of cells to spread and adhere onto a surface, whether it be a basement membrane, ECM, or tissue culture dish, is highly dependent on dynamic changes in the activation of the focal adhesion kinase (FAK) and Src kinase (22). FAK and Src are intimately linked to each other and activated via “outside-in” signals, through binding of ligands to cell surface receptors, leading to changes in cytoskeletal organization, cell motility, and adhesion (9). TNC is able to bind to receptors on the cell surface and regulate FAK activity and in turn has been shown to enhance Src activity in breast cancer (21, 23). However, the relationship between TNC and Src has not been discussed in Ewing sarcoma. Therefore, we hypothesized that TNC present in the TME from either tumor cell secretion or stromal cell secretion, may bind to the cell surface and thus be able to activate Src kinase in Ewing sarcoma. To test this hypothesis, TC32 and CHLA10 cells were treated with 3 uM recombinant TNC or vehicle control under unstressed conditions for 24 hours. At this concentration, there were no changes in cell adhesion to the tissue culture dish or changes in cell morphology. Cells were lysed and resulting lysates were analyzed by western blot for phosphorylation of Src on tyrosine

418 (activating) -TNC application led to significantly increased Src activation (**Figure 4.2A**).

As published previously, Ewing sarcoma cells exposed to serum starvation and hypoxia result in increased activation of Src (4). Here we now show that extrinsic TNC can also enhance Src phosphorylation. Therefore, we hypothesized that stress dependent activation of Src would be dependent, at least in part, on cell intrinsic expression of *TNC*. Knockdown of *TNC* using two short hairpins (shTNC3 and shTNC5) was first confirmed by qRT-PCR (**Figure 4.2B**). Here we found that knockdown of *TNC* decreased both baseline and stress-dependent induction of p-Src on tyrosine 418 in CHLA10 (**Figure 4.2C**). In conclusion, we show that both cell extrinsic and cell intrinsic production of TNC mediates 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 (12). Src phosphorylation at tyrosine 418 is a positive regulator in generating these structures in many cancers, including Ewing sarcoma (4). Additionally, *TNC* expression in cancer is associated with a more metastatic phenotype, specifically through increased expression at the invasive front of many solid tumors (24). As we have now shown that TNC can increase Src activation, we next sought to determine if exogenous TNC present in the TME could also further enhance invadopodia formation under stress. Ewing sarcoma cells were seeded onto Oregon green 488 labeled gelatin-coated chamber slides as previously described (4) and subjected to serum deprivation in addition to application of vehicle or 3 micro-Molar

TNC for 24 hours. Cells/slides were fixed and invadopodia/areas of matrix degradation were imaged (**Figure 4.3D**) and quantified (**Figure 4.3E**). TNC application resulted in a statistically significant increase in matrix degradation than serum starved control cells. Together these data support the hypothesis that increased TNC in the microenvironment activates Src and enhances invadopodia formation and surrounding matrix degradation in Ewing sarcoma.

Given our finding that TNC enhances invadopodia formation through p-Src in Ewing sarcoma and that knockdown of *TNC* partially blocks stress dependent Src activation, we next sought to determine if *TNC* is necessary for stress induced invadopodia. We hypothesized that stress dependent activation of invadopodia structures is mediated by the expression and subsequent secretion of TNC into the local TME. To test this hypothesis, control and *TNC* knockdown Ewing cells were exposed to serum deprivation and hypoxic conditions and were cultured on Oregon green 488 labeled gelatin-coated chamber slides. As shown (**Figure 4.3A-D**), loss of *TNC* significantly impeded stress induced activation of invadopodia, as indicated by loss of invasive structures and overall degradation. Based on these findings, we conclude that stress dependent activation of invadopodia is dependent on cell intrinsic *TNC* and TNC in the TME can enhance invadopodia formation, providing evidence for a link between Src, TNC, and the invasive phenotype.

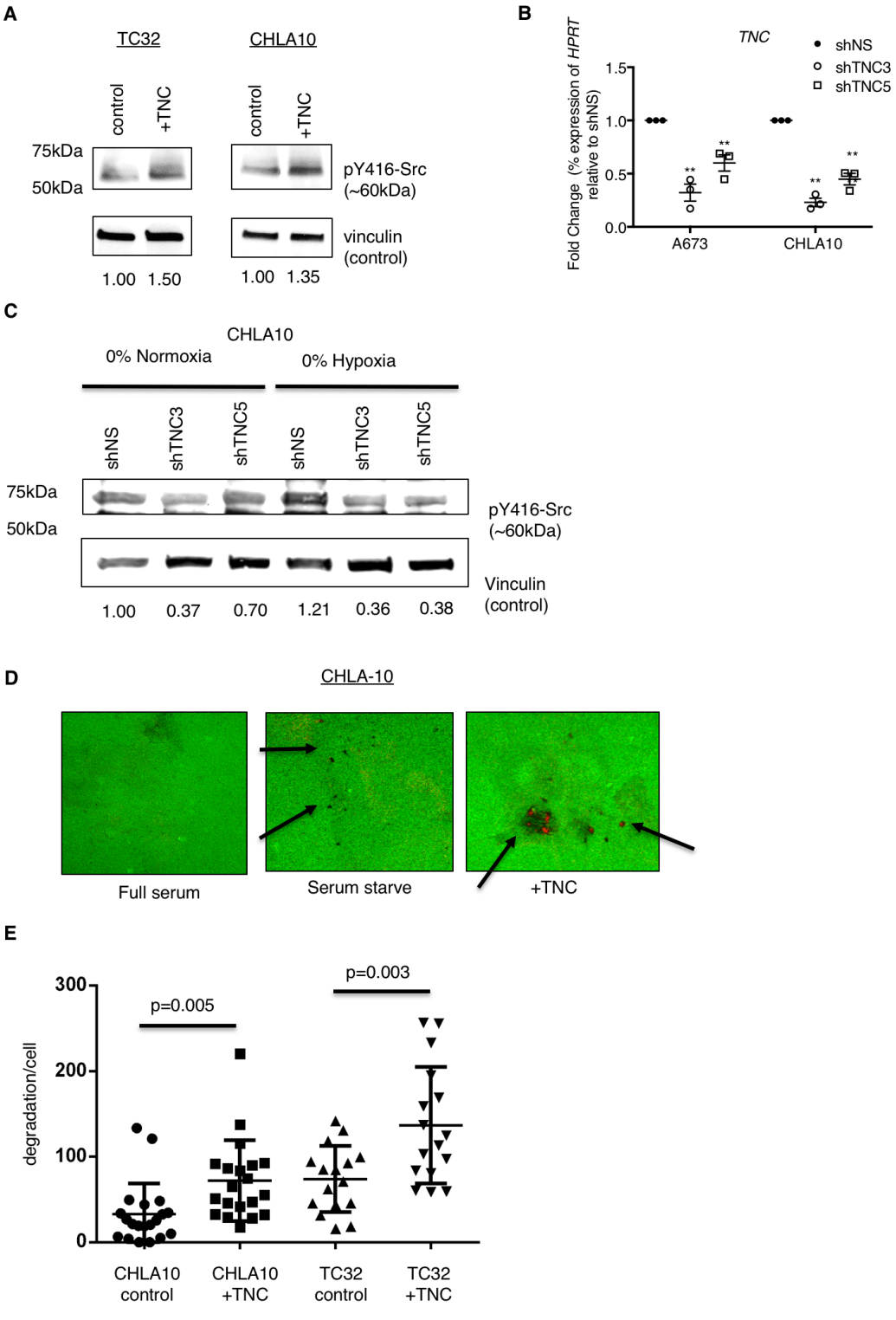
A
TC32
CHLA10
control
+TNC
control
+TNC
75kDa
50kDa
pY416-Src
(~60kDa)
vinculin
(control)
1.00 1.50
1.00 1.35
B
TNC
shNS
shTNC3
shTNC5
Fold Change (% expression of HPRT relative to shNS)
1.5
1.0
0.5
0.0
**
A673
CHLA10
C
CHLA10
0% Normoxia
0% Hypoxia
shNS
shTNC3
shTNC5
shNS
shTNC3
shTNC5
75kDa
50kDa
pY416-Src
(~60kDa)
Vinculin
(control)
1.00 0.37 0.70 1.21 0.36 0.38
D
CHLA-10
Full serum
Serum starve
+TNC
E
p=0.005
p=0.003
degradation/cell
300
200
100
0
CHLA10
control
CHLA10
+TNC
TC32
control
TC32
+TNC

Figure 4.2. Tenascin C increases Src phosphorylation and invadopodia formation in Ewing sarcoma. A. TC32 and CHLA10 Ewing sarcoma cells were treated with 3 micro-Molar recombinant tenascin C (TNC) or vehicle control for 24 hours. Lysates were prepared and subjected to western blot analysis for p-Y418 Src and vinculin (loading control). B. Ewing sarcoma cell lines CHLA10 and A673 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 was measured via western blot in control CHLA10 cells (shNS) or CHLA10 TNC knockdown (shTNC3 and shTNC5) cells in the presence of single stress (SS) or dual stress (SS + Hypoxia). Ewing cells were then 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. D. 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) (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. E. 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.

Stress induces Tenascin C expression in Ewing sarcoma

Here we show that stress-dependent induction of invadopodia is dependent on Src activation (4) and *TNC*. Our published studies showed that expression of *TNC* in Ewing sarcoma cells is heterogeneous and dynamic, and that it can be induced by canonical Wnt signaling (5). We wondered whether *TNC* expression was also dynamic in the presence of stress, explaining the dependency of stress-induced invadopodia formation on *TNC* expression. Ewing sarcoma cell lines, A673 and CHLA10 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 RT-PCR. Both single and dual stress conditions resulted in increased *TNC* expression in both cell lines (**Figure 4.1A**). 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, dependent on cell line, resulted in statistically significant increases in TNC protein (**Figure 4.1B, Figure 4.1C**). Therefore, like canonical Wnt/beta-catenin signaling, exposure of Ewing cells to hypoxia and serum deprivation increases *TNC* transcript and protein levels, suggesting that stress induced invadopodia formation occurs, at least in part, because of stress-induced expression of *TNC*.

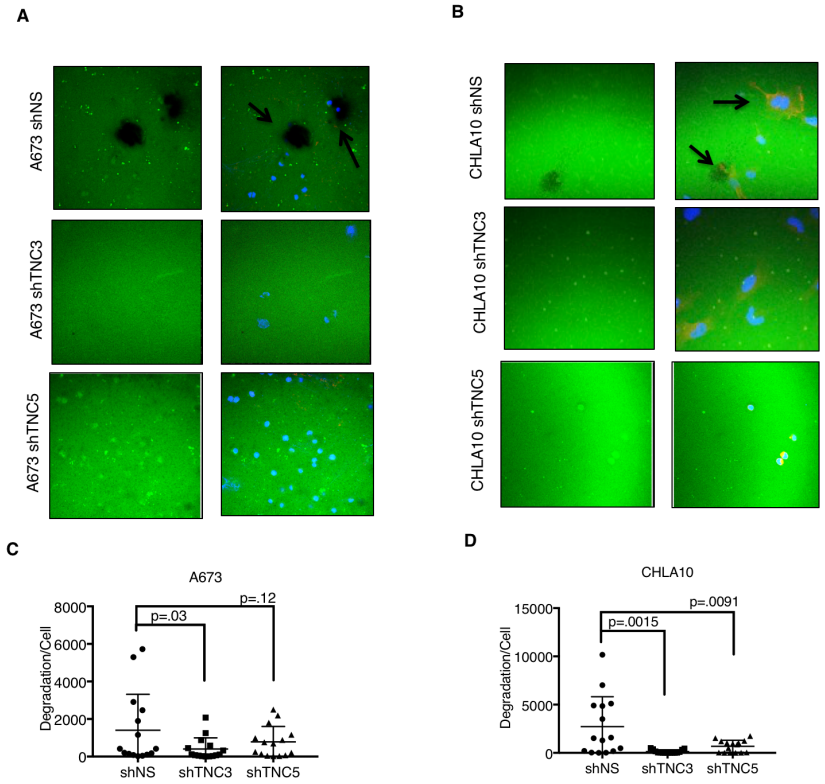

Figure 4.3. Knockdown of Tenascin C decreases invadopodia formation. A673 (A) and CHLA10 (B) were cultured for 24 hours on Oregon Green 488 labeled gelatin coated chamberslides. Cells were incubated on slides in 0% serum and hypoxia. Representative high-power field images are shown of gelatin (green only) and gelatin + cortactin (red) and DAPI (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. C&D. Degradation was quantified using ImageJ analysis to determine degradation/cell number for each image. Experiments were performed in triplicate and p-values were determined using student's t-tests and * indicates p<.05, ** indicates p<.005.

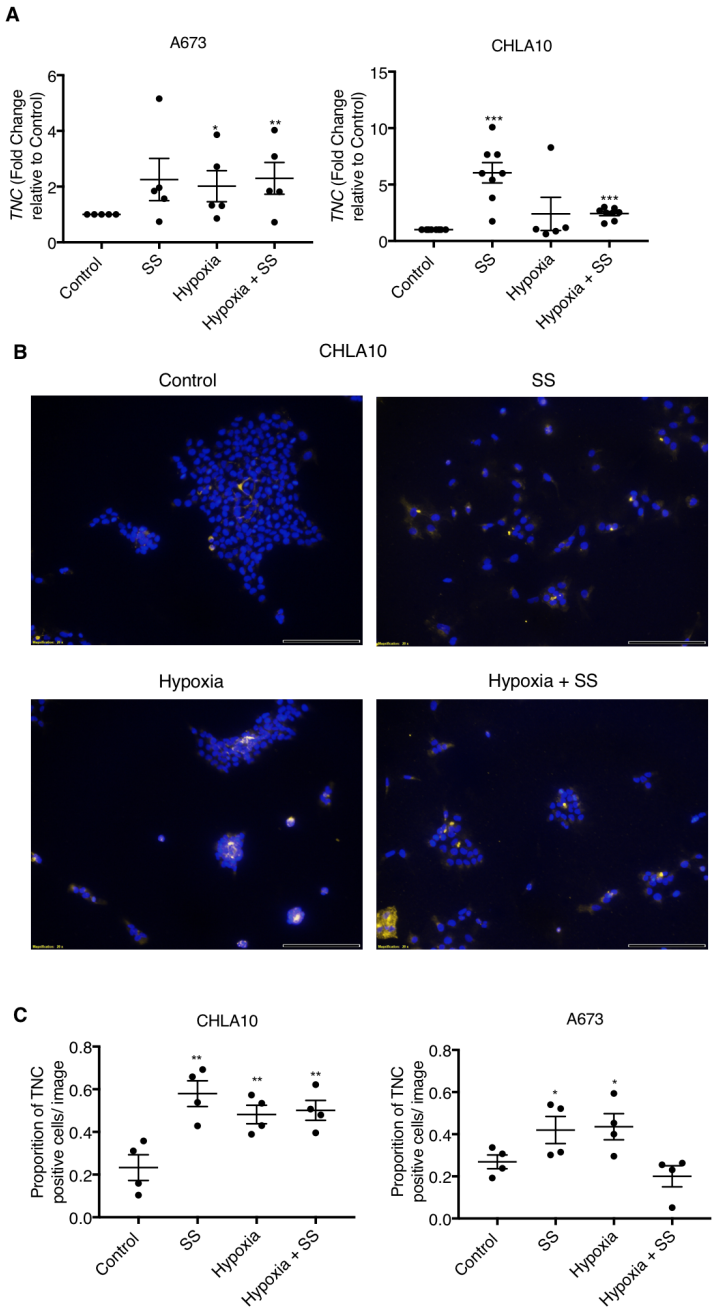

Figure 4.4. Stress induces Tenascin C expression in Ewing sarcoma. A. A673 and CHLA10 cells were cultured with full serum (10% FBS for A673, 20% FBS for CHLA10) in normoxia (control), 0% serum in normoxia (SS), full serum in hypoxia (Hypoxia), and 0% serum in hypoxia (Hypoxia + SS). Cells were collected 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. C. Proportion of cells that are positive for TNC in high power-field images in A673 and CHLA10. P-values were determined using student's t-tests and * indicates $p<.05$, ** indicates $p<.005$.

The Src inhibitor dasatinib decreases Tenascin C expression in Ewing sarcoma

Stress has now been shown to increase both Src activation and cell intrinsic expression of *TNC*, and stress-induced invadopodia formation is abrogated upon either *TNC* knockdown or inhibition of Src. Therefore, we sought to determine if stress dependent *TNC* expression in Ewing sarcoma cells was regulated by stress-dependent Src activation or vice versa. We first treated Ewing cells with nanomolar doses of dasatinib to block phosphorylation of Src (as described previously (4)) and measured baseline *TNC* expression to determine if Src activation regulates *TNC* in the absence of stress. Across three different cell lines, we observed a decrease in *TNC* expression in dasatinib treated cells as compared with vehicle treated cells (**Figure 4.5A**). This suggests, that activation of p-Src is upstream of cell intrinsic *TNC* expression.

Next, to determine whether stress-induced *TNC* expression is also mediated by Src, cells were cultured in full serum plus normoxia or no serum plus hypoxia 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 4.4**, and that this induction of *TNC* by stress is blocked by the addition of dasatinib (**Figure 4.5B**). Based on these findings, we conclude that both basal and stress induced *TNC* expression is dependent on Src activation.

Finally, we evaluated whether Src dependent activation of TNC expression was restricted to stress induction of *TNC*, or if *TNC* is dependent on Src when *TNC* is activated through other avenues. For example, we previously published that activation of the Wnt/beta-catenin pathway results in increased expression and secretion of TNC (5, 6). Src kinase has also been implicated in playing an important role in Wnt/beta-

catenin driven cancers (25-27). Therefore, we hypothesized that Wnt dependent expression and secretion of TNC is also dependent on activation of Src in Ewing sarcoma. Here we see that in A673 and CHLA10 cells, dasatinib blocks the Wnt dependent induction of *TNC* (**Figure 4.5C**). To determine whether or not Wnt induced TNC secretion was also dependent on p-Src, we stimulated Ewing cells with Wnt3a for three days and then added dasatinib for 48 hours prior to collecting secreted protein and checking for secreted protein levels via western blot. We see that in both A673 and CHLA10 activation of Wnt/beta-catenin through treatment with Wnt3a induces TNC secretion and this is blocked with the combination of both Wnt3a and Dasatinib. This data shows that cell intrinsic *TNC* expression in both Wnt induced and stress induced conditions is downstream of Src activation. Together, these data suggests that TNC and Src are linked by a feed forward loop where stress (and/or Wnt) induces Src activation followed by an increase in cell intrinsic *TNC* expression and formation of invadopodia. TNC can then be secreted into the TME, either by Ewing cells or stromal cells, and signal back to tumor cells to further induce Src activation and enhance invadopodia formation.

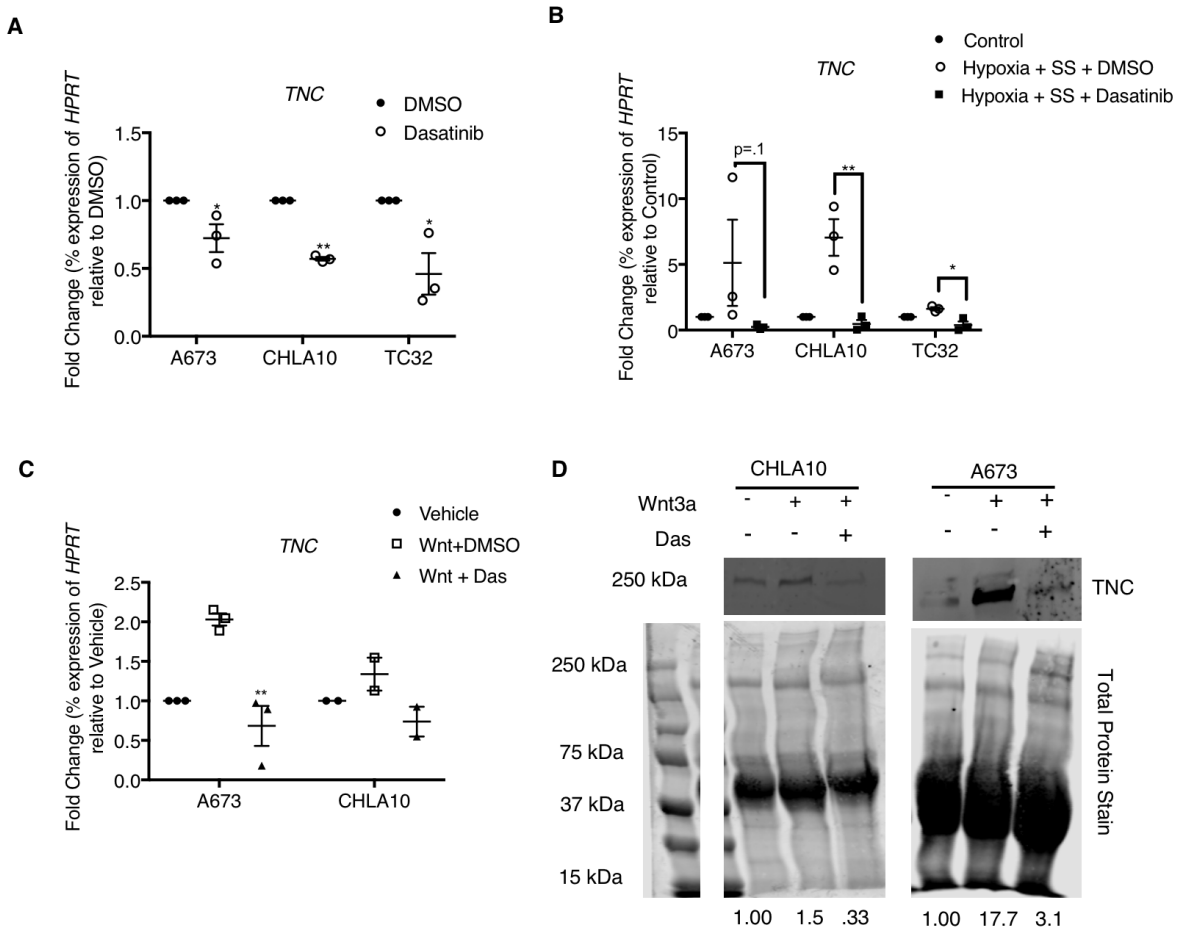

Figure 4.5. The Src inhibitor Dasatinib decreases Tenascin C expression and secretion in Ewing sarcoma. A. A673, CHLA10, and TC32 cells were treated with either vehicle control (DMSO) or 50 uM dasatinib for 24 hours prior to collecting to determine *TNC* expression via qRT-PCR. B. Cells were cultured 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. C. Cells were treated with Wnt3a (100 ng/mL) or vehicle (PBS) for 24 hours with or without 50 uM dasatinib. *TNC* expression was measured using qRT-PCR. D. 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 uM 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.

Discussion

Work presented here and published data has demonstrated the importance of tumor microenvionmental stress/Src phosphorylation mediated *TNC* expression on Ewing sarcoma cell aggressiveness through the generation of invadopodia and *in vivo* tumor lung seeding, respectively (4). We propose a model in which stress induces activation of Src kinase, resulting in increased *TNC* expression. Cell intrinsic and extrinsic TNC then promote a feed forward loop in which TNC increases Src activation and enhances invadopodia formation (**Figure 4.6**). For the first time, we have shown the significant intersection of the stress/p-Src/TNC pathways in dictating Ewing sarcoma invasive potential.

Here we show that TNC mediates two critical phenotypes important in cancer progression, cell-cell interactions and invasion. 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 (14, 16). 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 (16). Ewing sarcoma cells have been shown to secrete high amounts of fibronectin (6), implying that high amounts of 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 rather than cell spreading on the tissue culture plate. Although the exact mechanism through which this occurs has yet to be described and is outside the scope of the manuscript, 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.

Reduced *TNC* expression results in a decrease in efficiency to form invadopodia while addition of TNC enhances invadopodia formation in Ewing sarcoma. TNC has been shown to promote an invasive phenotype in a neuroendocrine tumor model (28) as well as promote a metastatic phenotype through increased migration and activation of the epithelial to mesenchymal transition (21). However, TNC has yet to be described to contribute to formation of the invasive structures known as invadopodia in cancer. Invadopodia are defined by two distinct processes, the formation of the actin rich structure which is dependent on Src and the ability to degrade the local ECM primarily mediated by MMPs and changes in ECM composition (12, 29). TNC can bind to both fibronectin and collagen fibrils as well as other ECM proteins to alter ECM composition or enhance degradation and can bind to integrins on the cell surface to mediate motility and adhesion in cancer (14, 17, 24). Although we have yet to show the exact mechanism through which TNC activates invadopodia, we hypothesize that TNC activates the invasive phenotype either through integrin dependent activation of Src to form the actin rich core or through direct interaction with ECM proteins in the TME resulting in matrix degradation. Together, the data shown here implies that production of TNC by both Ewing sarcoma cells as well as other stromal cells in the TME, promotes the invasive potential of Ewing sarcoma tumors.

We now have shown that enhanced *TNC* expression is one of the critical components of the Ewing cell response to TME-stress. *TNC* expression is controlled by mechanical stress, but this is the first study to link *TNC* to microenvironmental stress

that is not a result of mechanical alterations (30, 31). *TNC* is very lowly expressed in adult tissues outside of areas of wound repair (15). 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 have presented one avenue through which *TNC* expression could be increased and hypothesize that microenvironmental stresses, such as nutrient deprivation and hypoxia found in large, necrotic tumors contribute to the high expression of *TNC* in the stroma of solid tumors.

We used low nM doses of dasatinib (previously shown by us to block p-Src mediated invadopodia in Ewing sarcoma (4)) to also block stress-induced increases in *TNC* expression, suggesting a dependency of *TNC* expression on Src activation. We show that application of TNC enhances Src phosphorylation and loss of *TNC* expression inhibits stress dependent Src activation. Therefore, we propose a positive feed forward loop in which microenvironmental stresses induce Src activation which results in increased TNC expression and subsequent secretion. Both cell intrinsic and extrinsic TNC present in the TME can then further induce Src activation, presumably through integrin binding, leading to increased invasion. As shown in **Figure 4.6**, this loop is stimulated by extrinsic signals in the local microenvironment including stress and/or Wnt activation.

Interestingly, dasatinib also blocked Wnt-induced/non-stress mediated upregulation of *TNC*, suggesting that Src kinase may also be important in downstream Wnt signaling in Ewing sarcoma, although exact understanding of how Src kinase could be linked to canonical Wnt signaling is a remaining question to be answered. In fact,

there are reports of non-canonical Wnt signaling triggering the activation of Src (32). This finding suggests that there are multiple avenues that can affect Src phosphorylation and subsequently *TNC* expression in Ewing sarcoma to mediate a metastatic phenotype.

In conclusion, 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 inhibitors targeting the Wnt (33) pathway or Src alone (4, 34) may not prevent stress-mediated increases in *TNC* expression. In this situation, acute stress (hypoxia, nutrient deprivation) may provide a means by which cells could rapidly upregulate *TNC*, activate Src and promote invasive behavior.

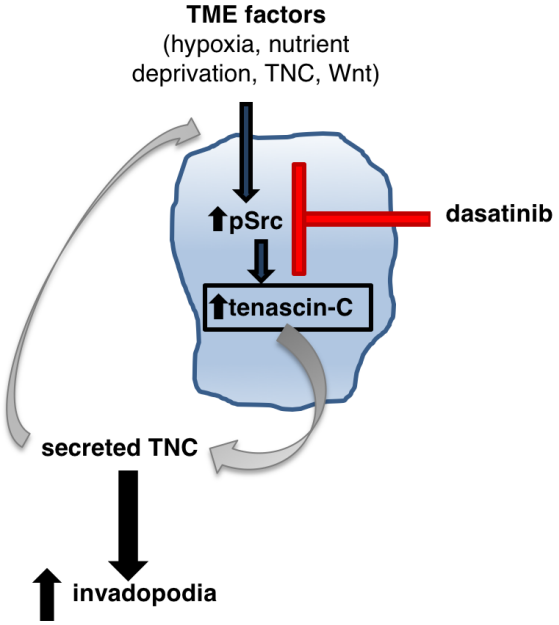

Figure 4.6. Visual Overview. Both tumor microenvironmental (TME) stress and Wnt can upregulate *TNC* expression in Ewing sarcoma cells and can be blocked by application of dasatinib. Increased exposure to or expression of TNC results in increased invasive potential via formation of invadopodia.

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 2mmol/L-glutamine (Life Technologies). CHLA10 was maintained in IMDM media (Fisher Scientific) supplemented with 20% FBS, 2mmol/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 were STR profiled and are mycoplasma negative. For hypoxia experiments, cells were cultured in 1% O2 as previously described (4).

Reagents

Dasatinib was a gift from the Leopold lab (Ann Arbor, MI) and used at a concentration of 50 uM. 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 (4) and Sigma TRCN0000230788 (shTNC) was used. Transduced cells were selected in puromycin (2 ug/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 three kilodalton 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(6). Antibodies were obtained from the following sources: pSrc (Tyr418 Cat # 44-660G Invitrogen/Thermo Fisher, Waltham, MA) 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 (4). Ewing tumor cells were cultured on the slides and following completion of the experiment, the cells/slides were fixed with paraformaldehyde, permeabilized with .1% Triton X, and quenched with 50mM 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 710 confocal microscope. Total degradation was quantified using ImageJ as described in (35) 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 $p < 0.05$ and ** indicates $p < 0.005$.

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Chapter 5: Conclusion and Future Directions

Ewing sarcoma is an aggressive bone and soft tissue tumor with peak incidence in adolescents and young adults, however it can present at any age (1). High dose chemotherapy combined with radiation and/or surgery has led to improved survival rates for patients with only primary tumors. In contrast, no improvement in the past 30 years has been made in treating the third of patients who go on to develop metastatic disease or relapse, most commonly in the form of metastatic disease (2). These patients have less than a 20% chance of 5 year survival and this is further decreased to a dismal 10% chance of survival for patients developing metastasis to the bone (1). Not only have we made no improvement in treatment of metastatic disease, high dose systemic chemotherapy given to all patients results in acute and long-term toxicities even long after they are cured of the disease (2). Future research should not only focus on finding better therapies for patients with metastatic disease but also on improving current treatment strategies to improve overall survival while decreasing long term toxicities.

Ewing sarcoma is driven by EWS-ETS fusion proteins, most commonly EWS-FLI1 identified in 85% of patients (3). However, as EWS-FLI1 is a transcription factor, it is an extremely poor drug target (4). EWS-FLI1 functions as a pioneer factor leading to

global reprogramming of the epigenome that alters gene expression to create an EWS-FLI1-dependent gene signature (5, 6). Failed targeting of the fusion itself, led to ongoing efforts to target direct protein interactions with RNA helicase A (RHA) that are thus far unsuccessful (7). Attempts to reverse the EWS-FLI1 gene signature through treatment with trabectedin that interferes with activated transcription has also been unsuccessful as a single agent in a phase II clinical trial (8). Focus has thus shifted from targeting EWS-FLI1 directly to targeting other pathways that are hijacked in Ewing tumors. Some examples of other approaches that are still being evaluated include CXCR4 inhibitors, lysine-specific demethylase 1 (LSD1) inhibitors, Poly (ADP-ribose) enzyme (PARP) inhibitors, anti-VEGF monoclonal antibodies, insulin-like growth factor-1 receptor (IGF-1R) inhibitors, and inhibitors of bone resorption (2, 4). It is imperative that we focus our efforts on understanding the biological drivers of both local and metastatic disease in hopes of exploiting new therapeutic targets such as the ones mentioned here to improve patient survival.

Notably, many of these potential therapeutic targets include pathways that alter tumor: TME interactions rather than tumor cells directly. In this thesis work, we propose a role for two more pathways affecting tumor: TME interactions to drive an aggressive phenotype, the Wnt/beta-catenin pathway and the TGF-beta pathway. We show that activation of the Wnt/beta-catenin pathway leads to increased secretion of ECM components like TNC, a matricellular protein critical in dictating tumor: TME crosstalk through increased angiogenesis and invadopodia formation (9). We then show that Wnt sensitizes cells to activation of the TGF-beta pathway through derepression of the EWS-FLI1 target, *TGFBR2*. Expression of the pro-metastatic *TNC* is then shown to be

dependent on the cooperation between Wnt and TGF-beta signaling. Furthermore, we show a connection between *TNC* expression and activation of Src kinase, a key accelerator of the invasive phenotype in Ewing sarcoma. Both Wnt and TGF-beta have independently been shown to mediate Src kinase in other cancer types, suggesting that all three of these pathways are linked in driving Ewing sarcoma metastasis (10, 11). Preclinical studies using a porcupine inhibitor, resulting in decreased Wnt ligand secretion, have shown decreased tumor growth in xenograft models, revealing that targeting the Wnt pathway may be a potential avenue to improve patient survival (12). Based on these findings, we hypothesize that inhibiting a combination of Wnt, TGF-beta, and Src signaling could decrease the metastatic phenotype and improve overall survival for metastatic patients.

Wnt and TGF-beta remodel the Tumor Microenvironment

Invasion and Matrix Degradation

Here we are the first to show that activation of the Wnt pathway sensitizes cells to activation of the TGF-beta pathway, resulting in increased expression of the pro-angiogenic *TNC*. However, activation of both the Wnt and TGF-beta pathways alter many cellular processes and it is highly unlikely that angiogenesis is the only change in the TME that occurs upon pathway activation (13, 14). A prominent role for TGF-beta is in driving an invasive phenotype and specifically, heterogeneous activation of TGF-beta at the invasive front has been identified in squamous cell carcinoma (15). Activation of the TGF-beta pathway was originally found to act as a tumor suppressor in primary xenograft models of Ewing sarcoma (16). However, recent understanding of the

conflicting roles of TGF-beta throughout tumor progression in carcinoma models suggests that TGF-beta may also be context dependent in Ewing sarcoma (17).

Wnt pathway activation results in increased migration but is unable to induce invadopodia formation and matrix degradation *in vitro* (18). Therefore, we hypothesized that Wnt activated cells are primed to respond to TGF-beta ligands in the TME, creating a pro-invasive phenotype and contributing to the metastatic phenotype observed in Wnt activated Ewing sarcoma xenografts. In support of this hypothesis, preliminary data in 2D shows that cells pretreated with Wnt3a prior to activation of the TGF-beta pathway have increased matrix degradation and matrix remodeling in comparison to either ligand alone (**Figure 5.1**). Next, we hope to explore the role of Wnt and TGF-beta activation in a 3D collagen matrix and evaluate both cell motility as well as matrix degradation to definitively determine if Wnt and TGF-beta promote invasion.

Extracellular Matrix Remodeling

A major finding of this dissertation work is that activation of the Wnt pathway leads to increased expression and secretion of proteins involved in ECM organization and composition. We then go on to show that Wnt-dependent induction of these ECM genes is dependent on the TGF-beta pathway. We therefore hypothesize that cooperation between these pathways drives changes in structure and composition of the local TME.

In order to accurately show the contribution of Ewing sarcoma cells to ECM remodeling and ECM composition we need to utilize 3D systems that allow for cells to grow in the context of a local ECM. To begin addressing this question, we collaborated

with Dr. Joerg Lahann's group in the chemical engineering department to use their 3D fibronectin (FN) coated scaffold (19). Here, scaffolds are coated using phase separation technology to allow for FN to reveal its FNIII sites and be in its extended form vs. the globular form found in tissue culture dishes (20, 21). This allows for the FN to form in it's physiological state and results in an almost uniform 3D matrix (**Figure 5.2A**). We can then culture cells on this 3D matrix and look for changes in the FN matrix.

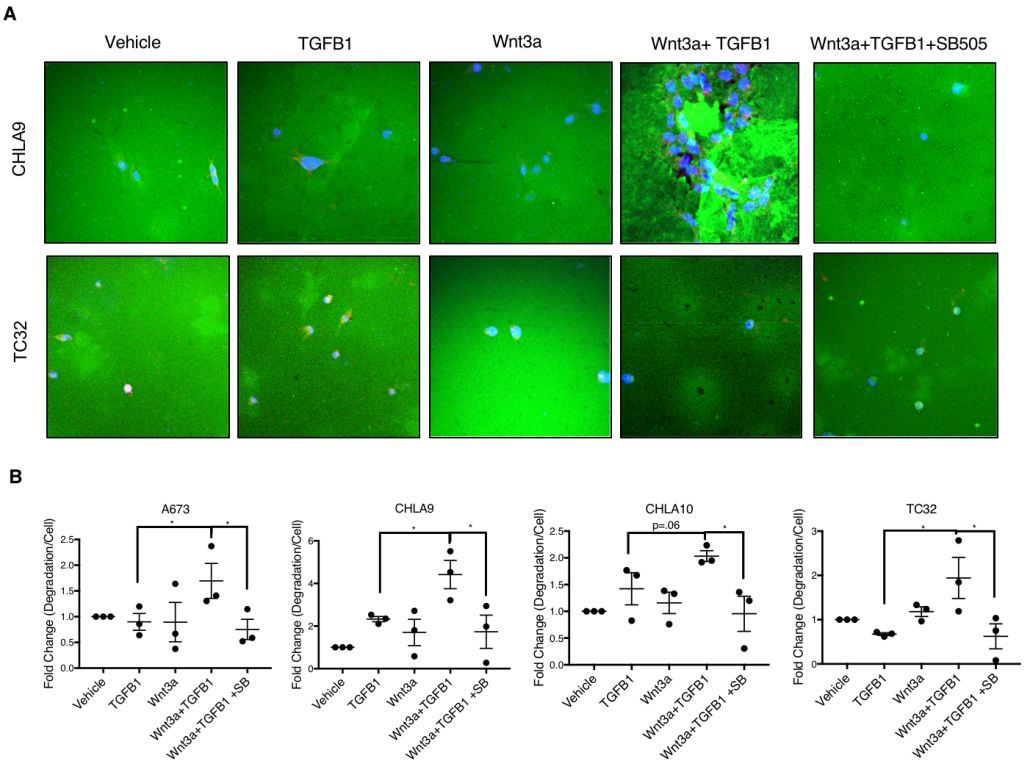

Figure 5.1 Wnt and TGF-beta cooperate to promote matrix degradation. A. Cells are cultured in the presence or absence of Wnt and/or TGFB1 on FITC-labeled gelatin (Green). Matrix degradation and remodeling is indicated by black holes in gelatin and changes in gelatin color intensity. **B.** Matrix degradation is quantified across three replicates with a minimum of 5 high power field images and averaged. Total degradation is normalized to cell number present in each image. Statistically significant increases in matrix degradation is observed in cells treated with both Wnt3a + TGFB1. This is blocked with addition of the TGF-beta inhibitor, SB505124 (indicated as SB in figure). Student's t-tests were used to determine statistical significance. * indicates $p<0.05$

To begin these studies, we focused our efforts specifically on understanding the effect of *TNC* on the FN matrix. Throughout this thesis, we have discussed the importance of *TNC* in mediating the metastatic phenotype and also note that *TNC* is one of the many ECM proteins dependent on cooperation between Wnt and TGF-beta signaling. TNC is a key mediator of tumor: TME interactions and primarily binds to fibronectin via FNIII domains. This extended form of FN coated on the FN scaffold is particularly useful for studying the effect of TNC on cell-matrix crosstalk due to the repetitive FNIII binding domains present in the TNC protein (22). Therefore, we hypothesized that expression of *TNC* alters how Ewing sarcoma cells remodel the FN matrix. Ewing sarcoma cells cultured on these FN scaffolds results in massive changes in FN structure and leads to formation of a "holy" matrix (**Figure 5.2B-C**). In the presence of *TNC* knockdown, Ewing sarcoma cells can no longer alter the FN matrix and the matrix has a more uniform appearance (**Figure 5.2B-C**). These findings lead us to the conclusion that expression of *TNC* is critical in driving tumor cell – matrix interactions and alters how tumor cells are able to remodel the ECM.

Here, we present one preliminary finding in which a Wnt and TGF-beta target, *TNC*, is important in mediating remodeling of a FN matrix. Future experiments should address whether or not this is specific to TNC production, or does activation of Wnt and TGF-beta together cooperate to change how the ECM is structured. Additionally, culturing cells on a more physiological ECM containing collagen I, as is found in the bone TME, the primary site for many Ewing sarcoma tumors, would better model the TME interactions and ECM remodeling occurring in the bone. Finally, development of *in vivo* models to examine the effect of Ewing sarcoma cells on local ECM remodeling in

the primary tumor itself would help us better understand the role of tumor cells in altering composition and structure of the local ECM. If our findings are consistent with our preliminary data, we can conclude that activation of both Wnt and TGF-beta is critical in driving changes in the TME and matrix remodeling to promote the invasive phenotype discussed earlier.

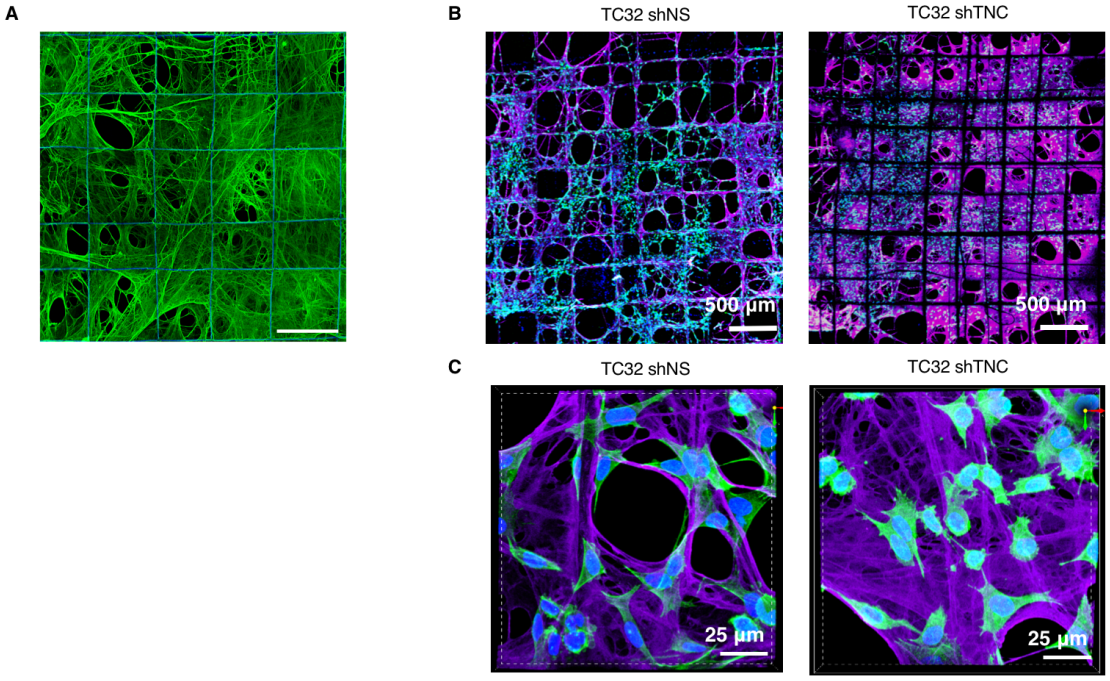

Figure 5.2 Loss of TNC alters fibronectin matrix remodeling. A. 3D scaffold coated with fibronectin using phase separation technology gives rise to a mostly uniform 3D fibronectin coated matrix (green = fibronectin). 10X (**B**) and 60X (**C**) images of Ewing sarcoma TC32 cells containing either non silencing (shNS) or TNC targeted hairpin (shTNC) cultured on the fibronectin matrix for 4 days before fixation and immunofluorescent staining. Fibronectin shown in purple, actin shown in green, and nuclei indicated by DAPI staining. Loss of TNC changes the fibronectin matrix and decreases holy matrix observed in shNS.

Changing the bone tumor microenvironment

One of the drivers of Ewing sarcoma progression is dictated by the osteolytic environment found in the bone. In the bone microenvironment, osteoblast differentiation

and activation lead to osteoclast activation resulting in bone resorption and osteolysis. During bone resorption, growth factors sequestered in the matrix, such as TGF-beta ligands, are released into the microenvironment, increasing their bioavailability (23). Both Wnt and TGF-beta ligands have been shown to signal to tumor cells, such as osteosarcoma cells, resulting in increased secretion of proteins that further enhance this "vicious cycle" between osteoblasts and osteoclasts in the bone (23, 24). One such secreted protein previously shown to be upregulated in pro-metastatic Ewing sarcoma cells is parathyroid hormone related protein (PTHrP) (25). PTHrP is a TGF-beta target, therefore we hypothesize that Wnt and TGF-beta treated cells would have increased expression and subsequent secretion of proteins, such as PTHrP, leading to increased osteolysis either through direct activation of osteoclasts or indirect activation through osteoblasts. Preliminary exploration of this pathway revealed an increase in *PTHrP* expression in cells treated with Wnt3a and TGFB1 (**Figure 5.3A**). This increase is abrogated with application of the TGF-beta inhibitor, SB505124, implying that *PTHrP* expression is dependent on TGF-beta (**Figure 5.3B**). We are currently testing the hypothesis that increased expression of *PTHrP* translates into increased secretion of PTHrP followed by activation of osteoclasts. Ongoing studies include culturing osteoclast precursor cells with conditioned media from Ewing sarcoma cells treated with Wnt3a and TGFB1 to determine if proteins secreted from Wnt and TGF-beta activated cells can induce osteoclast activity. Finally, if our findings support our hypothesis, we will specifically test the contribution of PTHrP to the osteolytic phenotype through gain and loss of function studies *in vivo*. Further investigation into the crosstalk between

tumor cell expression of osteoblast and osteoclast activating factors will help describe the role of Wnt and TGF-beta activation on changing the bone TME.

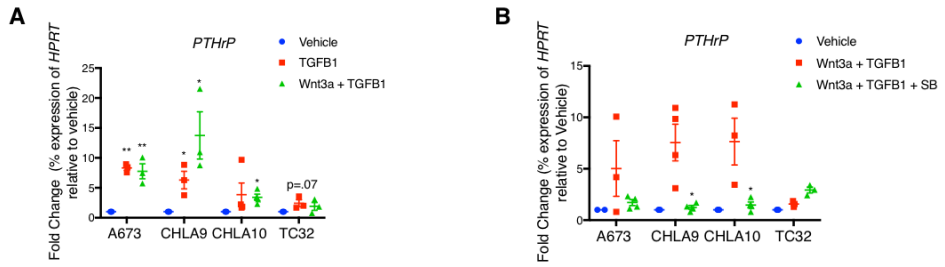

Figure 5.3 Wnt and TGF-beta activated cells have increased expression of *PTHrP*. A. Ewing sarcoma cell lines were treated with TGFB1 alone or Wnt and TGFB1 in combination. Increased expression of *PTHrP* was observed with both treatments. **B.** Inhibition of TGF-beta with the TGF-beta inhibitor SB505124 (SB in figure) blocked induction of PTHrP expression with both Wnt3a and TGFB1 in 3 of 4 cell lines. All experiments were performed in triplicate and student's t-tests were used to determine statistical significance. * indicates $p<0.05$ and ** indicates $p<0.005$.

Cell cooperation and metastasis

Interestingly, we also highlighted that only subpopulations of Ewing cells are able to activate the Wnt and/ or TGF-beta pathways, but we do not yet understand the distinction between a Wnt and/or TGF-beta responsive vs. unresponsive cell. Recent literature on tumor heterogeneity has supported a theory referred to as clonal cooperation, in which genetically, epigenetically, and/or phenotypically different subpopulations within a tumor cooperate to promote tumor progression (26-28). For example, a zebrafish melanoma xenograft model was used to identify inherently invasive and non-invasive cells that invade the local TME together in what is described as "cooperative invasion" (29). In our studies we show distinct subpopulations of Wnt responsive, TGF-beta responsive, and Wnt and TGF-beta responsive cells, however activation of these smaller subpopulations are enough to induce a metastatic cell state. Therefore, we hypothesize that these subpopulations are able to cooperate to promote

these metastatic phenotypes and that both unresponsive and responsive cells are necessary for Ewing sarcoma progression. To test this hypothesis, functional assays to measure each subpopulations' effect on angiogenesis and invasion will be performed. Further understanding of the individual roles of each subpopulation will elucidate the precise function that Wnt and TGF-beta responsive cells have within a Ewing sarcoma tumor.

It is clear based on the studies presented here that the Wnt and TGF-beta pathways are playing a critical role in dictating cell phenotype. We reported clear changes in angiogenesis and future studies will focus on determining if there is a role for these pathways in mediating invasion and/ or the osteolytic TME in the bone. Both of these pathways and all of the processes discussed regulate tumor: TME interactions, further proving the importance of the TME in driving Ewing sarcoma progression.

TNC as an "Achilles heel"

Prior to the work discussed in this thesis, expression of *TNC* in Ewing sarcoma cells had been shown to be necessary for lung engraftment in a xenograft model. Additionally, Wnt/beta-catenin was published to increase *TNC* expression in Ewing sarcoma cell lines (18). Here, we build on this finding and show that Wnt activation also increases TNC secretion. Although loss of *TNC* blocked the metastatic phenotype, the exact mechanism through which *TNC* alters metastatic potential has yet to be elucidated.

TNC is a matricellular protein whose primary role is in mediating adhesion of cells to the local ECM. TNC is unique in that it can bind and activate integrins on the cell

surface while also binding to fibronectin, collagen, and other ECM proteins (30). High expression of *TNC* in solid tumors is correlated with poor prognosis and has been published to promote invasion, changes in adhesive properties, and angiogenesis (31). In this thesis, we have discussed at length the specific involvement of TNC in Ewing sarcoma and show that it contributes to both the invasive and angiogenic phenotypes.

We also show that *TNC* expression is dependent on activation of Wnt, TGF-beta, and Src, and that expression is dynamic under conditions of microenvironmental stresses. This supports the hypothesis that *TNC* can be upregulated in Ewing sarcoma via multiple avenues, dependent on the state of the TME. Additionally, Wnt/beta-catenin mediated angiogenesis and Src dependent invadopodia formation were both blocked with loss of *TNC* expression or function. These are two critical phenotypes in driving metastasis as discussed in the introduction. The first step of metastasis relies on the ability of cells to invade into the local ECM and one of the rate limiting steps of metastasis is the ability to induce angiogenesis to create a pro-tumorigenic TME at a distal site (32). We have now shown that TNC, which can be induced through Wnt, Src, TGF-beta, and microenvironmental stress, is critical for both of these key metastatic processes. In addition to these processes found to be affected by *TNC* in Ewing sarcoma, *TNC* can also alter cell adhesion and structure of the extracellular matrix to be conducive to tumor growth and metastasis (33). Although we have not discussed these other two phenotypes in Ewing sarcoma, it is very likely that TNC may be involved in either or both of those processes.

Based on the prominent role that TNC is playing in Ewing sarcoma, we hypothesize that targeting TNC directly, rather than the Wnt, TGF-beta, and/or Src

pathways individually, may in fact block invasion, angiogenesis, and matrix remodeling and thus reverse the harmful effects of activating these pathways. Additionally, *TNC* is only expressed in conditions of wound repair but otherwise is not expressed in the body (30). This makes TNC a prime target for Ewing sarcoma in order to block tumor growth and minimize off target effects and long-term toxicities commonly affecting Ewing sarcoma patients. Attempts to block *TNC* expression and function through inhibitory compounds, antibodies, and small peptides are currently ongoing and future studies should focus on testing these compounds in Ewing sarcoma models (34). Although we recognize that many other secreted proteins could be contributing to the angiogenic and invasive phenotypes, these studies make it clear that TNC is a very large factor. As a downstream target of all the pathways discussed here that promote Ewing sarcoma progression, TNC may in fact be an “Achilles heel” in metastatic Ewing sarcoma and exploitation of this target could be crucial to improving overall patient survival.

Conclusions

In conclusion, this thesis addresses the contribution of Wnt/beta-catenin signaling to changes in tumor: TME interactions, revealing a novel link between Wnt and TGF-beta, and further highlighting the importance of TNC in Ewing sarcoma (**Figure 5.4**). We discuss multiple routes through which *TNC* expression can be altered based on the TME, supporting previous findings in Ewing sarcoma cells that these cells are extremely plastic and dynamic (18, 35, 36). Of note, we also see heterogeneous responses to these various microenvironmental stimuli accompanied by significant changes in phenotype, suggesting the potential for these heterogeneous populations to

cooperate in driving metastasis. Ewing sarcoma tumors will not be eradicated simply by targeting the tumor cell, rather we should focus on targeting the tumor: TME interactions that promote disease progression. We have identified that two key signaling pathways cooperate to promote metastatic phenotypes and changes in tumor: TME crosstalk. Furthermore, we find that activation of the pathways discussed converge on TNC, therefore we propose that targeting TNC, a protein expressed by both tumor cells and stromal cells in the TME, will improve overall survival in Ewing sarcoma patients.

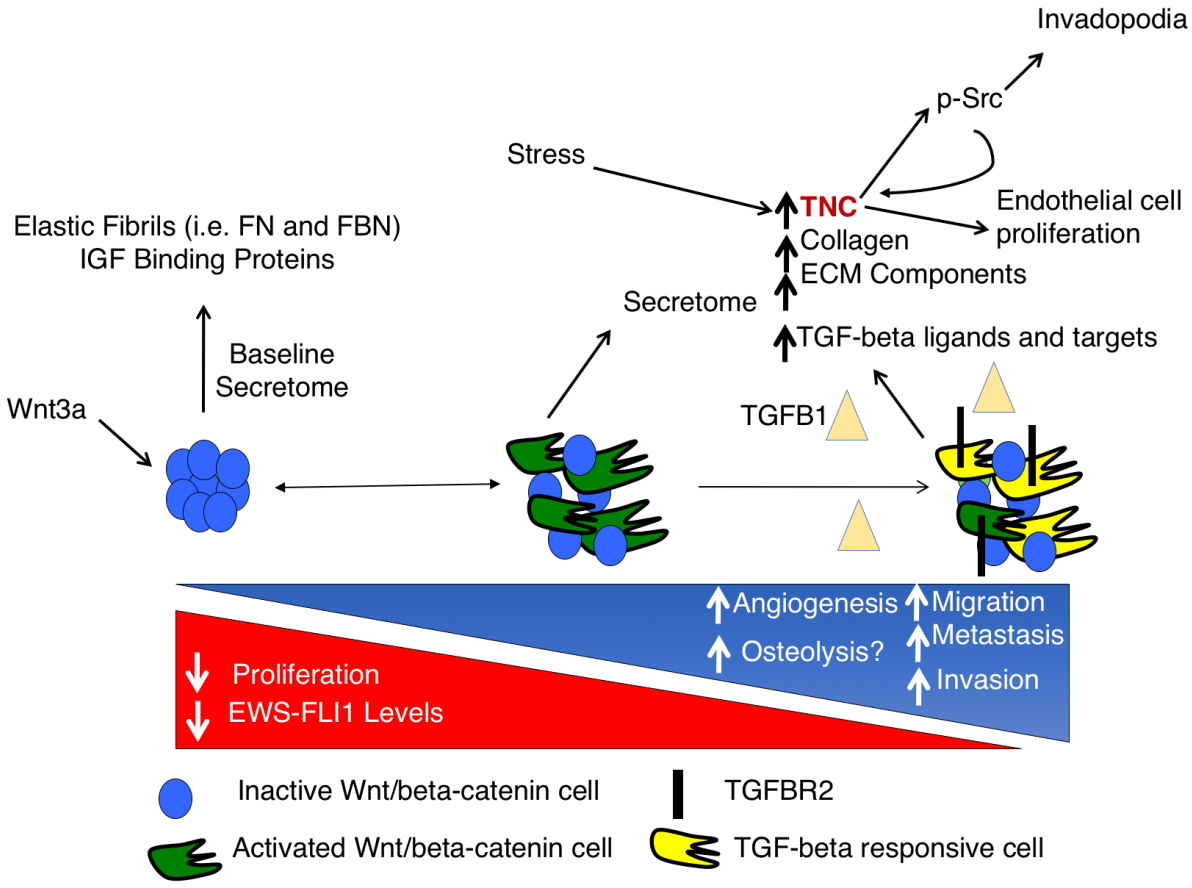

Figure 5.4 Thesis Summary. Cooperation between Wnt/beta-catenin and TGF-beta signaling induce a metastatic phenotype via increased expression and secretion of TNC. TNC induces endothelial cell proliferation and activates Src kinase to increase invadopodia formation.

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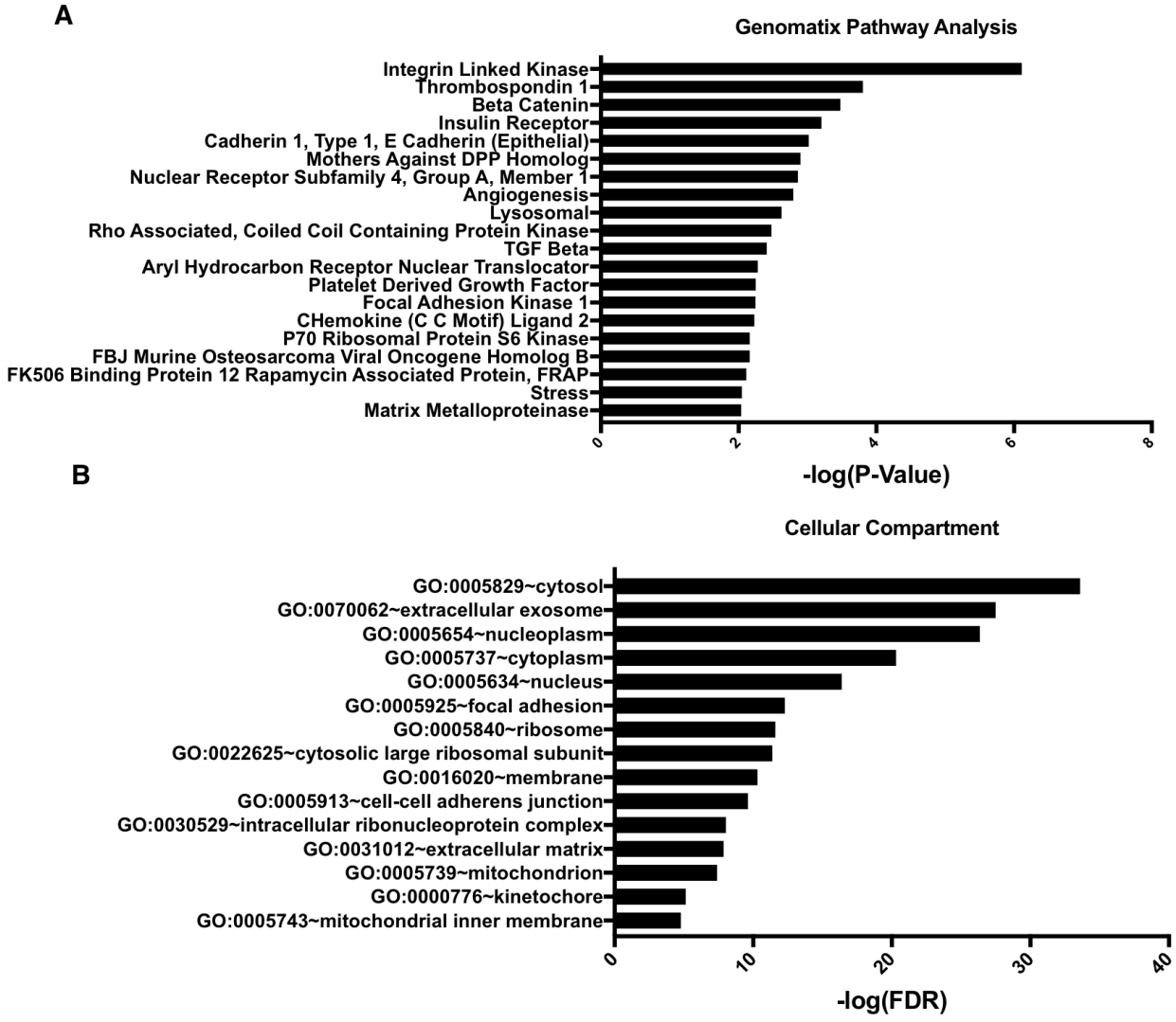
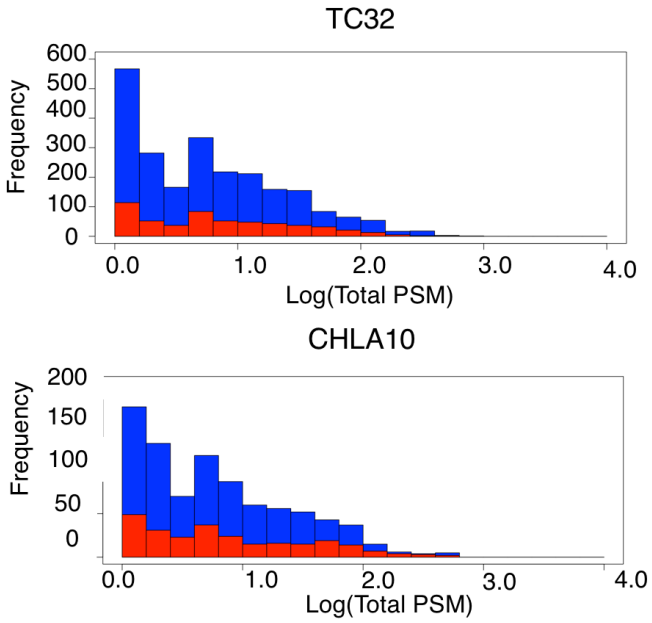
Appendices

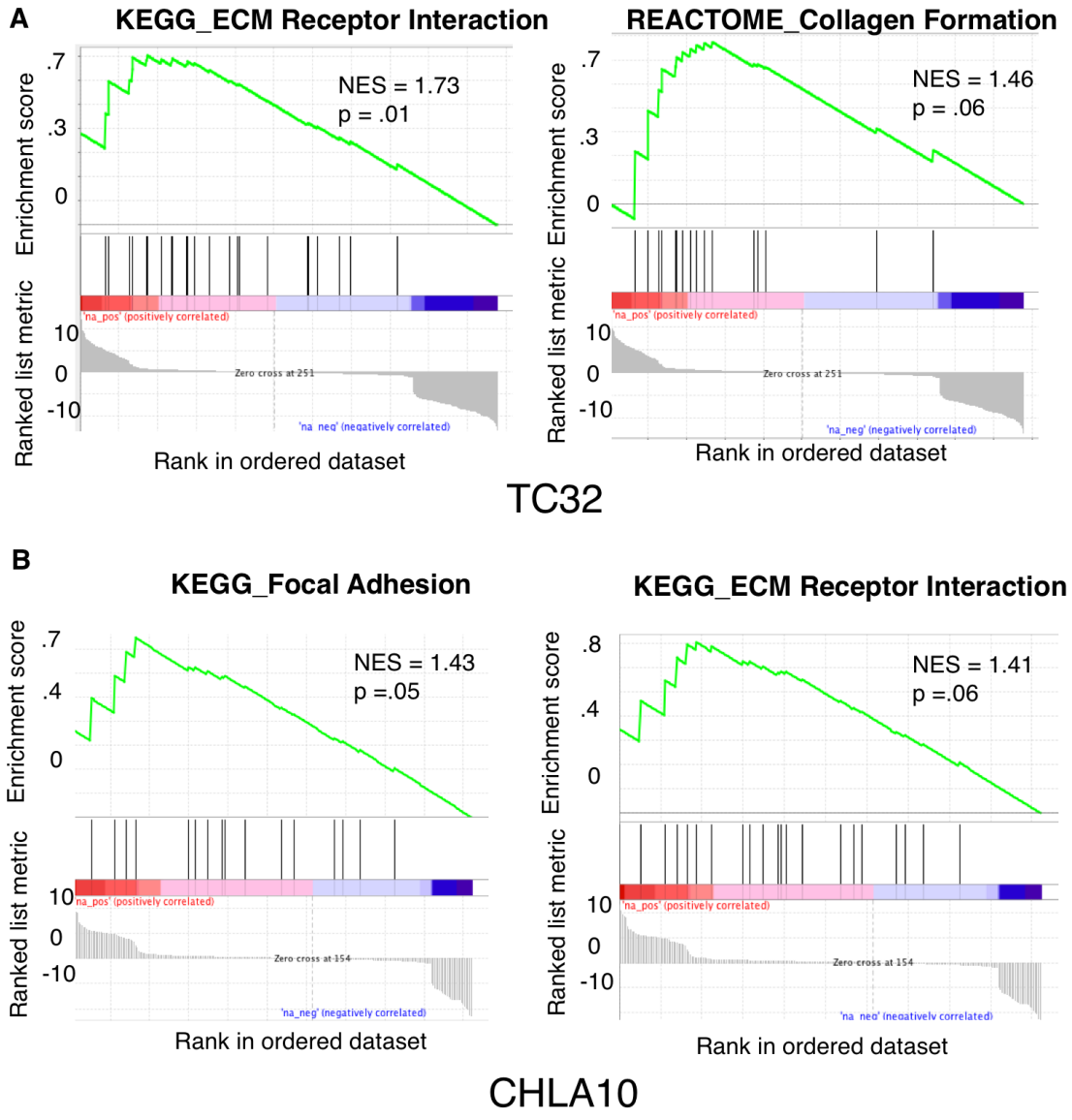
Appendix 1: Supplemental Figures



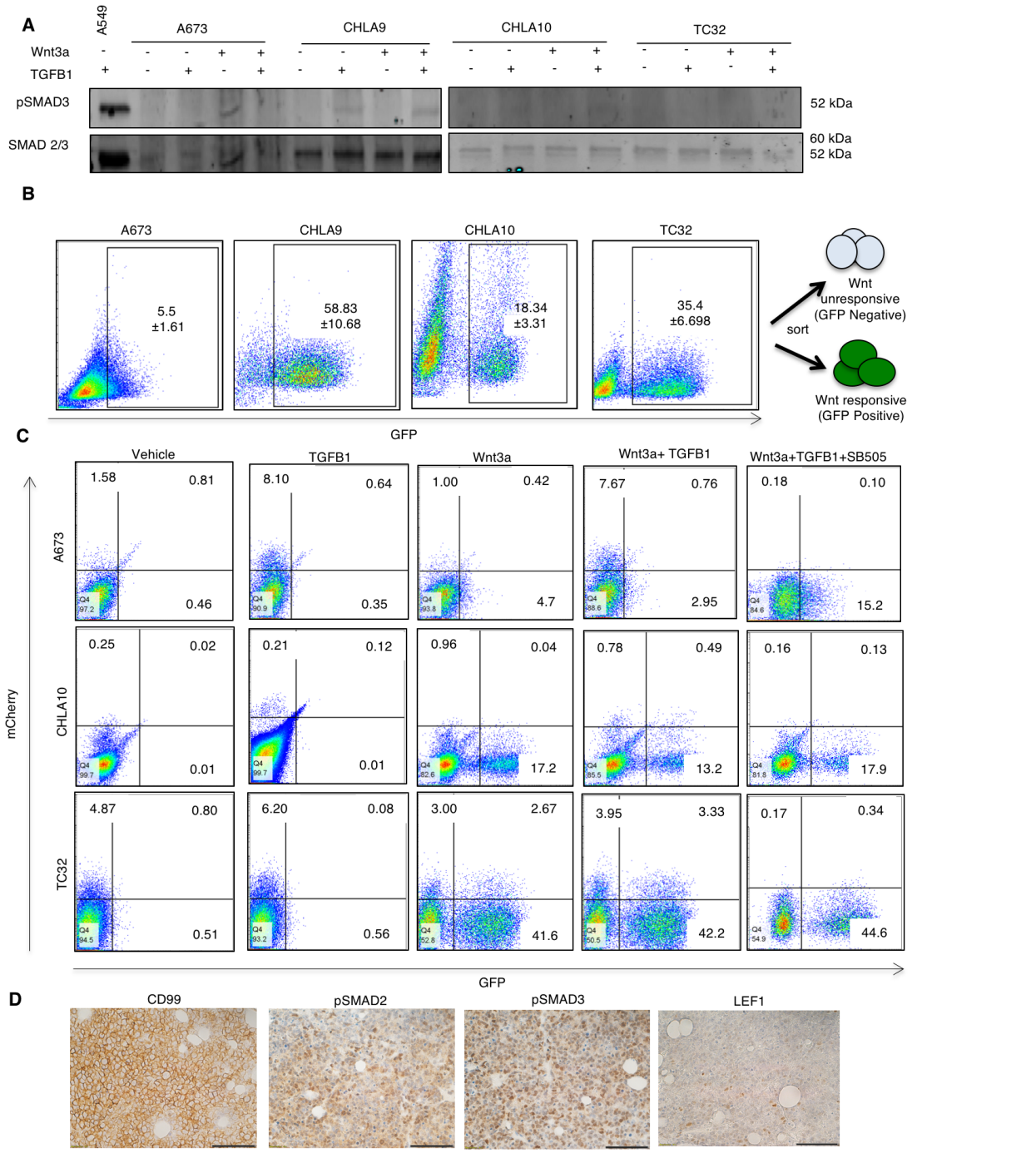
Supplemental Figure 2.1. Wnt activated transcripts are enriched for tumor: tumor microenvironment interactions. A. Pathway analysis using the Genomatix Pathway System tool on genes identified to be upregulated in response to Wnt3a alone or Wnt3a + Rspondin2 in Pedersen et al., 2016. All statistically significant pathways are shown. B. Gene ontology analysis using DAVID on the same list of genes as in A. Cellular compartment is shown along with corresponding -log(FDR).



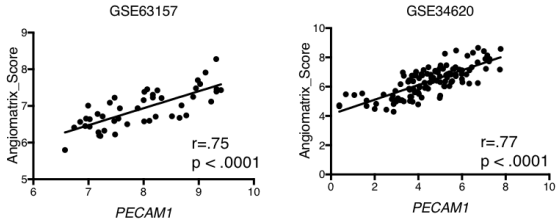
Supplemental Figure 2.2. Secreted proteins are not biased towards low or high abundant proteins. Secreted proteins (red) are found to have the same distribution of PSMs as all proteins identified (blue) in the analysis in both TC32 and CHLA10.



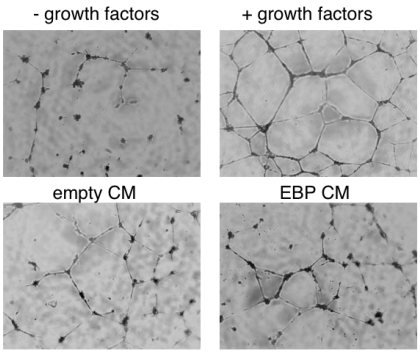
Supplemental Figure 2.3. The Wnt dependent secretome is enriched for extracellular matrix components. A. GSEA analysis of ranked list of proteins identified in the secretome in TC32. Ranking is done based on fold change between Wnt treated and vehicle treated samples. The top 2 gene sets are shown. B. GSEA analysis of ranked list of proteins identified in the secretome in CHLA10. The top 2 gene sets are shown.



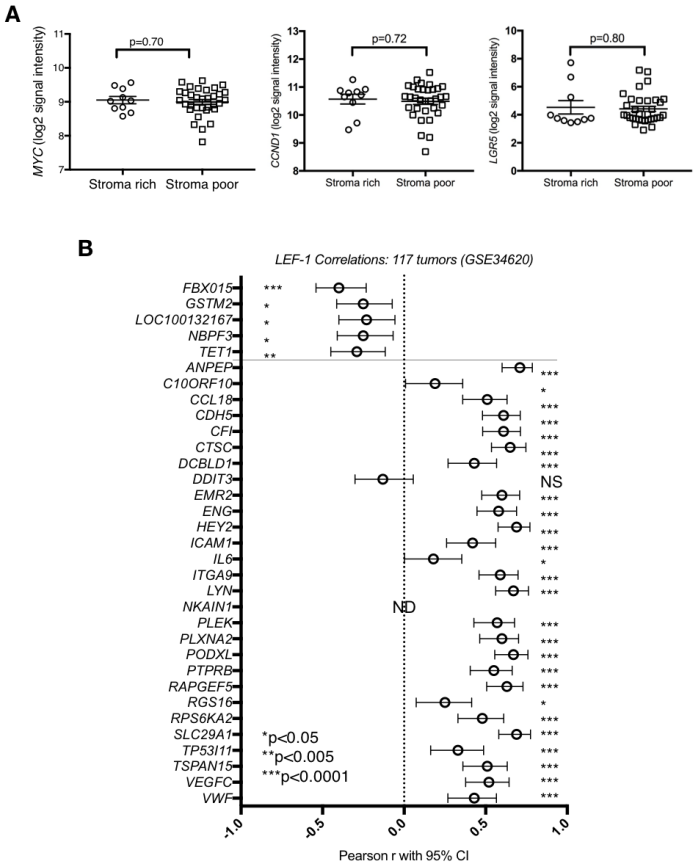
Supplemental Figure 3.1. Ewing sarcoma cells are heterogeneous in response to Wnt and TGF-beta. A. Western blot of pSMAD3 in cells that were pretreated with vehicle or Wnt3a before treatment with TGFB1. B. Cells containing the TCF/LEF-GFP reporter were treated with Wnt3a for 48 hours prior to sorting on GFP. Average percentage of GFP positive cells and standard deviation for each cell line are shown in each plot. C. Dual reporter Ewing sarcoma cells containing a TCF/LEF-GFP reporter and SBE-mCherry reporter were pretreated with Wnt3a prior to treatment with TGFB1 +/- SB505124. FACS plots show percentage of GFP positive (Wnt responsive), mCherry positive (TGF-beta responsive), and double positive (Wnt and TGF-beta responsive) cells in a representative experiment in A673, CHLA10, and TC32. D. IHC staining for CD99, pSMAD2, pSMAD3, and LEF1 in a representative A673 femur xenograft.



Supplemental Figure 3.2. The AngioMatrix score is correlated with angiogenesis in Ewing sarcoma. Correlation of the AngioMatrix score (as calculated in Figure 3.5E and 3.5F) with log2(signal intensity) of PECAM1 in two independent patient cohorts of N=46 (GSE63157) and N=117 (GSE34620). All correlation coefficients shown are Pearson r values and p-values are determined using a student's t-test.



Supplemental Figure 3.3. EBP Conditioned media does not enhance vascular morphogenesis. Conditioned media from EBP cells does not enhance branching morphogenesis of cultured HUVECs (bottom). Control HUVECs with/without endothelial growth factor media are shown for comparison (top).



Supplemental Figure 3.4. The AngioMatrix is correlated with poor prognosis in patients. A. Classic Wnt target genes, MYC, CCND1, and LGR5, are not induced by Wnt3a in Ewing sarcoma and are not differentially expressed between stroma poor (N=33) and stroma rich tumors (N=10). P-values were determined using student's t-test. B. Pearson correlations (r) of LEF1 expression (error bars: 95% confidence intervals (CI)) with 33 prognostic signature genes in 117 primary, localized patient tumor biopsies. Top 5 genes were identified as good prognosis biomarkers, whereas high expression of bottom 28 genes was associated with poor prognosis. ND indicates no data available for indicated gene. NS indicates not significant.

Appendix 2: Author Contributions

Chapter 2

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Chapter 3

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Chapter 4

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Appendix 3: List of Oligonucleotide Primers

Primer	Sequence	Figure
HPRT Forward	5' TGACACTGGCAAAACAATGCA 3'	2.3, 2.5, 3.1, 3.4, 3.6, 4.2, 4.4, 4.5
HPRT Reverse	5' GGTCCTTTTCACCAGCAAGCT 3'	2.3, 2.5, 3.1, 3.4, 3.6, 4.2, 4.4, 4.5
TNC Forward	5' GCAGCTCCACACTCCAGGTA 3'	2.5, 3.4, 3.6, 4.2, 4.4, 4.5
TNC Reverse	5' TTCAGCAGAATTGGGGATTT 3'	2.5, 3.4, 3.6, 4.2, 4.4, 4.5
COL1A1 Forward	5' CTGGACCTAAAGGTGCTGCT 3	2.5, 3.4
COL1A1 Reverse	5' GCTCCAGCCTCTCCATCTTT 3'	2.5, 3.4
CTGF Forward	5' CTGGTCCAGACCACAGAGTG 3'	3.4
CTGF Reverse	5' GCACTTTTTGCCCTTCTTAATGT 3'	3.4
TGFBR2 Forward	5' AATGTGAAGGTGTGGAGAC 3'	3.1, 3.6
TGFBR2 Reverse	5' GGTAGGCAGTGGAAAGAG 3'	3.1, 3.6
LEF1 Forward	5' TGGATCTCTTTCTCCACCCA 3'	2.3, 3.6
LEF1 Reverse	5' CACTGTAAGTGATGAGGGGG 3'	2.3, 3.6
AXIN2 Forward	5' AAGTGCAAACTTTCGCCAAC 3'	2.3, 3.6
AXIN2 Reverse	5' ACAGGATCGCTCCTCTTGAA 3'	2.3, 3.6
NKD1 Forward	5' TCGCCGGGATAGAAAACTACA 3'	2.3
NKD1 Reverse	5' CAGTTCTGACTTCTGGGCCAC 3'	2.3

Table A.3 List of oligonucleotide primers used in each figure.