ROLE OF TGF-β SIGNALING IN CARCINOMA CELL MIGRATION AND TUMOR PROGRESSION

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cancer Biology

August, 2012

Nashville, Tennessee

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To my loving parents who never cease to provide incredible support, strength, and encouragement. Throughout this journey, they have comforted me in the valleys, rejoiced with me at the peaks, and always believed in me. Without them, this academic journey would not have been possible, and for this, I am forever thankful.

ACKNOWLEDGEMENTS

This endeavor was graciously made possible through the financial support of the Department of Defense Breast Cancer Research Program Pre-Doctoral Award (W81XWH-11-1-0066). Additionally, this project was funded through NIH grants CA085492 and CA102162 (awarded to Harold Moses) and NIH grants CA120711 and CA143081 (awarded to Andries Zijlstra). I thank the members of my dissertation committee for their helpful suggestions and guidance during my graduate school career. Drs. Hal Moses, Al Reynolds, Andries Zijlstra, and Rebecca Cook are all talented scientists who believed in me, helped me to think scientifically, and assisted in broadening the scope of my project.

Thank you to all members of my Moses lab family. It has been a great pleasure working with and learning about them over these past several years. Their guidance and assistance have been key in completing this dissertation work, both scientifically and personally. I am thankful for their continual support and will miss them in more ways than I can count. I am also appreciative of Michael Pickup, for without him, this journey would have been infinitely more difficult. He always offered his shoulder on which to cry or laughter when needed. I especially thank Hal, my mentor. Hal has seen me both fail and succeed, and I appreciate the constant belief he had in my ultimate success. No matter my moments of irrationality or excitement, his even-keeled nature brought perspective to each situation. He has shown me by example why he is not only a good scientist but a good person as well.

I also would like to thank the Zijlstra lab for their patience in helping me with all of my chicken embryo experiments. Andries Zijlstra has been exceedingly gracious in taking me in as one of his own students, despite his popularity with students and busy schedule. I am extremely

thankful for his guidance, encouragement, and expertise. I would also like to thank Trenis Palmer for his rationality, support, friendship, and laughter each and every time we worked together.

More than anyone, I thank my family and friends who have been the rock on which I have leaned. They have been ears for my complaints and eyes for me to see that completion of this project was possible. Their encouragement and kind words gave me confidence each time an experiment proved difficult. Thank you, thank you, thank you!

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ALK-5	Activin receptor-like kinase 5
α-SMA	Alpha-smooth muscle actin
CAM	Chorioallantoic membrane of a chicken embryo
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CTL	Cytotoxic T lymphocyte
Dab-2	Disabled-2
$dnT\beta RII^{-rtTA}$	Dominant-negative type II TGF-β receptor unable to be expressed
$dnT\beta RII^{+rtTA}$	Dominant-negative type II TGF-β receptor and rtTA transgenes expressed
DMEM	Dulbecco's Modified Eagle's Medium
DOX	Doxycycline
ECM	Extracellular matrix
EGFP	Enhanced green fluorescent protein
EMT	Epithelial-to-mesenchymal transition
FBS	Fetal bovine serum
FSP1	Fibroblast-specific protein 1
HCl	Hydrochloric acid
I-Smads	Inhibitory Smads
LAP	Latency-associated peptide
LCM	Laser capture microdissection
LTBP	Latent TGF-β-binding protein
MDSCs	Myeloid-derived suppressor cells
MET	Mesenchymal-to-epithelial transition
MMTV	Mouse mammary tumor virus
MSI	Microsatellite instability
NBF	Neutral buffered formalin
NK	Natural killer
PI3K	Phosphatidylinositol 3-kinase
PyVmT	Polyoma virus middle T antigen
qPCR	Quantitative polymerase chain reaction
RII	Type II TGF-β receptor functional construct
R-Smads	Receptor-activated Smads
rtTA	Reverse tetracycline-dependent transactivator
SARA	Smad anchor for receptor activation
SBE	Smad-binding element
sTβRII	Soluble type II TGF-β receptor
sTβRIII	Soluble type III TGF-β receptor
TAK1	TGF-β activated kinase 1
TβRI	Type I TGF-β receptor
TβRII	Type II TGF-β receptor
$T\beta RII^{fl/fl}$	Type II TGF-β receptor floxed control
TβRII KO	Type II TGF-β receptor knockout
TβRIII	Type III TGF-β receptor

TGF-β	Transforming growth factor-beta
VEGF	Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

Overview

Transforming growth factor-beta (TGF-β) is a pleiotropic cytokine engaged in numerous roles in normal cellular maintenance and cancer. TGF-β signaling is frequently dysregulated in human cancer, making this pathway a focal point of tumorigenic modulation. The dogmatic role of TGF-β is as a tumor suppressor of epithelial behavior during tumor initiation and early progression. In later stages of progression, however, TGF-β assumes a role in tumor promotion. Pro-tumorigenic effects are coordinated autonomously, as well as through paracrine microenvironmental interactions. Study of these interactions has been the subject of investigation during the course of the past decade. Additionally, a more thorough understanding of the temporal switch between TGF-β as a tumor suppressor or promoter is of great therapeutic interest. Our results presented herein explore the contributions of tumor-stromal interactions and temporal switching to mammary tumor progression *in vivo*. Specifically, we have associated loss of carcinoma TGF-β signaling with fibroblast-induction of collective cell migration. Investigation of single cell versus collective cell migration was pursued through intravital imaging, gene expression analysis, and study of the metastatic cascade. Our study of temporal switching implicates TGF-β as an early tumor suppressor prior to or at tumor onset. Our combined results identify collective cell migration and suppression of TGF-β signaling as mediators of enhanced dissemination and metastasis.

TGF-β signal transduction

Three TGF-β ligand isoforms (TGF-β1, TGF-β2, and TGF-β3) are secreted from cells into the extracellular matrix in a biologically inactive form (latent TGF-β). The latent TGF-β complex consists of one of three latent TGF-β-binding protein (LTBP) family members, a TGF-β propeptide dimer, also known as latency-associated peptide (LAP), and a mature TGF-β homodimeric protein (Derynck et al., 1985; Saharinen and Keski-Oja, 2000). LTBP and LAP are bound through disulfide bonds, while LAP is found in noncovalent association with the mature TGF-β homodimer. Latent TGF-β is activated via dissociation of LAP from mature TGF-β, thereby unmasking receptor-binding epitopes of the TGF-β ligand. Dissociation and subsequent activation mainly occurs via proteolytic cleavage, such as through plasmin, but it is also evident through latent TGF-β interaction with thrombospondin-1 or $\alpha_v\beta_6$ integrin (Dabovic and Rifkin, 2008). In this way, active TGF-β ligands are capable of initiating downstream signaling through type I, II, and III receptors (TβRI, TβRII, and TβRIII, respectively).

TGF-β transmembrane receptors have short extracellular cysteine-rich domains and are phosphorylated on intracellular serine and threonine kinase residues. TGF-β1 and TGF-β3 homodimeric ligands, disulfide-linked, bind TβRII with high affinity (Cheifetz et al., 1988). TGF-β2 homodimeric ligand is unable to bind TβRII in the absence of accessory receptor TβRIII, also called betaglycan (Lopez-Casillas et al., 1993). It has been reported that TGF-β1/2 heterodimers are capable of engaging receptor, although to a lesser degree than homodimeric TGF-β1 ligand, which is the most abundant TGF-β isoform (Attisano and Wrana, 2002; Cheifetz et al., 1988). In the presence of ligand-bound TβRII, heterotetramers are formed at the cell surface between TβRI and TβRII homodimers, both of which are required for TGF-β signaling (Wrana et al., 1992; Wrana et al., 1994). This heteromeric complex facilitates TβRII phosphorylation of TβRI (ALK-5)

on its cytosolic glycine-serine-rich sequence, thereby conformationally activating TβRI kinase signaling. TGF-β effector signaling through this activated heteromeric complex occurs through both Smad-dependent and -independent mechanisms. In this way, TGF-β ligands have pleiotropic effects on a wide array of cellular processes, including proliferation, apoptosis, differentiation, motility, and development.

Canonically, TGF-β effector signaling is mediated by receptor-activated Smads (R-Smads), Smad2 and Smad3, which are directly phosphorylated by activated TβRI (Abdollah et al., 1997; Attisano and Wrana, 2002; Souchelnytskyi et al., 1997). Several adaptor proteins, such as Smad anchor for receptor activation (SARA) and Disabled-2 (Dab-2), facilitate access of the R-Smads to the receptor complex (Hocevar et al., 2001; Tsukazaki et al., 1998). SARA enhances R-Smad-receptor binding and is required for efficient Smad recruitment and activation, while Dab-2 is required for functional TGF-β signaling. Phosphorylated R-Smads couple with the common mediator Smad4 in a trimeric complex, which is translocated to the nucleus for transcriptional regulation of target genes (Feng and Derynck, 2005; Kawabata et al., 1998). This occurs through Smad complex interactions with transcription factors, as well as transcriptional coactivators and repressors, at a defined tandem repeats of a DNA sequence termed the Smad-binding element (SBE) (Johnson et al., 1999; Zawel et al., 1998). At the SBE, Smad3 and Smad4 of the activated Smad complex bind and stimulate transcriptional regulation. Common TGF-β transcriptional target genes, such as *PAI-1*, *JunB*, $p15^{Ink4B}$, and $p21^{Cip1}$ genes, typically have growth inhibitory functions (Dennler et al., 1998; Feng and Derynck, 2005; Feng et al., 2000; Jonk et al., 1998; Pardali et al., 2000). Just as there is a mechanism for Smad-dependent transcription, there is also a TGF-β-inducible feedback mechanism for inhibition of this process via inhibitory Smads (I-Smads), Smad6 and Smad7. I-Smads disrupt Smad-dependent TGF-β signaling by competing

with R-Smads for association with TβRI (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997).

TGF-β effector signaling is also non-canonically mediated through receptor-activated, Smad-independent mechanisms. Smad-independent activation of MAP kinases and Rho-like GTPases occurs through RhoA, Cdc42, Rac1, and TGF-β activated kinase 1 (TAK1) downstream signaling pathways that regulate cytoskeletal organization, cell adhesion, epithelial-to-mesenchymal transition (EMT), and motility (Bhowmick et al., 2001; Edlund et al., 2002; Wilkes et al., 2003). TGF-β can also induce the MAP kinase pathway, via JNK and Erk activity, to regulate Smad-independent transcription (Atfi et al., 1997; Hartsough and Mulder, 1995). In addition to Smad-dependent growth inhibition, Smad-independent growth inhibition can occur through phosphatidylinositol 3-kinase (PI3K) signaling (Bakin et al., 2000; Yi et al., 2005). Both Smad-dependent and -independent signaling networks are intricately regulated to provide distinct mechanisms of cellular behavior (Derynck and Zhang, 2003; Moustakas and Heldin, 2005; Siegel and Massague, 2003). The balance of these networks is responsible for the cell- and tissue-specific TGF-β effects still under investigation.

Alteration of the TGF-β signaling pathway in human cancer

The pleiotropic roles of TGF-β in cellular processes render the effects of TGF-β signaling diverse and highly context-dependent in both homeostatic and cancer cells. Studies have suggested a growth inhibitory, tumor suppressive role for TGF-β in epithelial tumor initiation and development; however, in late stages of tumor progression, TGF-β serves in a tumor promoting capacity. Consequently, various aspects of the TGF-β signaling pathway in human cancer are altered throughout the continuum of tumor progression, leading to variable impacts

on patient prognosis. Activating alterations, such as amplification or overexpression, are associated with TGF-β ligands and *Smad7* (Bierie and Moses, 2006a, b; Levy and Hill, 2006). In contrast, inactivating alterations, such as mutations, deletions, and downregulation, have been identified for *TGFBR1*, *TGFBR2*, *SMAD2*, and *SMAD4*.

TGF-β1 overexpression has been associated with breast, colon, esophageal, gastric, hepatocellular, lung, and pancreatic cancers (Bierie and Moses, 2006a; Levy and Hill, 2006). Ligand overexpression in these cancers correlates with tumor progression, metastasis, angiogenesis, and poor prognostic outcome. A *TGFB1* mutation, resulting in heightened TGF-β1 serum levels, has been associated with a lower risk of breast cancer, suggesting a tumor suppressive role of TGF-β1 in early tumor development (Gobbi et al., 1999; Ziv et al., 2001). In contrast, in invasive breast carcinoma, epithelial TGF-β1 and TGF-β2 were overexpressed in 67% and 76% of cases, respectively, and associated with areas of high microvessel density (de Jong et al., 1998a, b). TGF-β1 overexpression has also been evidenced in advanced colorectal cancer as well as in prostate cancer in which overexpression is associated with angiogenesis, metastasis, and poor patient prognosis (Tsushima et al., 1996; Wikstrom et al., 1998). In a prostate cancer study, it was also found that TGF-β1 overexpression correlated with loss of TβRII expression, correlating with decreased patient survival (Wikstrom et al., 1998). These studies lend support to the tumor promoting role of TGF-β ligands in late stages of tumor progression.

TGFBR2 was found to harbor mutations in microsatellite stable human colon cancer cells as well as those laden with high rates of microsatellite instability (MSI) (Grady et al., 1999; Markowitz et al., 1995). It has further been shown that MSI is associated with *TGFBR2* mutations in the 10-bp adenine repeat (polyA_{10}) region (Bacon et al., 2001). In breast cancer, *TGFBR2* MSI or mutation frequency each occurs in approximately 10% of sporadic breast cancers

(Levy and Hill, 2006; Seitz et al., 2003; Takenoshita et al., 1998; Tomita et al., 1999). Although *TGFBR2* is genetically stable in 90% of all breast cancers, deregulation of TβRII expression is commonly noted. Low levels of TβRII expression correlated with an increased risk of breast cancer for women with breast epithelial hyperplastic lesions lacking atypia, while loss of TβRII expression correlated with increased tumor grade (Gobbi et al., 2000; Gobbi et al., 1999). These studies support the tumor suppressive role of TGF-β signaling during early stages of tumor initiation and growth. Additionally, both epigenetic silencing and transcriptional repression are causes of *TGFBR1* and *TGFBR2* downregulation (Hinshelwood et al., 2007; Kang et al., 1999; Kim et al., 2000). These causes of *TGFBR1* and *TGFBR2* downregulation could explain tumor-associated TGF-β resistance. Mutation of *TGFBR1*, resulting in diminished receptor activity, has also been found in association with metastatic breast cancer (Chen et al., 1998b). In addition to epithelial regulation of *TGFBR2*, one study correlated overexpressed stromal TβRII with poor prognosis in breast cancer (Barlow et al., 2003). This implicates TGF-β signaling in the tumor microenvironment, and not strictly the epithelium, in cancer progression. It also suggests a role for stromal TGF-β regulation of the adjacent tumor cell population.

Mutation and loss of heterozygosity for *SMAD2* and *SMAD4* has been observed most commonly in colon cancer and up to 90% of pancreatic tumors (Akhurst and Derynck, 2001). In contrast, *SMAD4* mutations are rare (12%) in breast cancer (Levy and Hill, 2006). The fact that *SMAD* mutations are typically less common than receptor mutations may be due to compensation effects of Smad2, which is non-requisite, by Smad3 during TGF-β signaling mediation (Akhurst and Derynck, 2001; Brown et al., 2007). The importance of TGF-β signaling mediation by Smad3 could therefore explain why *SMAD3* mutations have not been identified in human tumors.

TGF-β signaling effects during homeostasis, tumor initiation and progression, and metastasis

TGF-β was initially identified as a soluble factor capable of stimulating and transforming normal fibroblasts (Moses et al., 1981; Roberts et al., 1981). It was then observed that TGF-β1 inhibited growth of epithelial cells, suggesting a role in tumor suppression (Moses et al., 1985; Roberts et al., 1985; Tucker et al., 1984). Antiproliferative TGF-β signaling activity and cytostatic maintenance remain functions of TGF-β important in combating tumorigenesis and progression. These are manifested in TGF-β induction of growth arrest and apoptosis. In epithelial cells, TGF-β induces G_1 phase cell cycle arrest in a Smad-dependent fashion during homeostasis. This occurs via transcriptional expression of cyclin-dependent kinase (CDK) inhibitors (*p15*^{Ink4b}, *p21*^{Cip1}) and transcriptional repression of transcription factors driving cellular proliferation (*c-Myc*, *Id1*, *Id2*) (Feng et al., 2000; Pardali et al., 2000; Siegel and Massague, 2003). TGF-β exerts its apoptotic effects through Smad-dependent expression of *SHIP*, *TIEG*, and *DAPK*, all apoptotic-promoting proteins (Siegel and Massague, 2008). Other mechanisms of TGF-β-mediated apoptosis, potentially Smad-independent, are currently being investigated.

In addition to cytostatic responses induced by TGF-β, homeostatic TGF-β signaling has been further implicated in numerous other cellular processes as a pleiotropic cytokine. Its functions include roles in differentiation, development, cell adhesion, migration, angiogenesis, and immune regulation (Derynck and Miyazono, 2008). Given that TGF-β is a master regulator of epithelial function, its roles specifically in cell adhesion and migration are important in understanding how this function is exploited in cancer progression. It has frequently been shown that TGF-β enhances migration of normal and transformed epithelial cells, including mammary, prostate, lung, and pancreatic cells (Ellenrieder et al., 2001; Festuccia et al., 1999; Mooradian et al., 1992; Muraoka et al., 2002). It has been demonstrated that this induced

migration disrupts junctional complexes, which serve as epithelial cell-cell contacts important for cellular communication, adhesion, and polarity (Coradini et al., 2011). At apico-lateral tight junctions, Par6, a regulator of epithelial polarity and tight junction assembly, constitutively interacts with TβRI (Ozdamar et al., 2005). Upon ligand binding, TβRI thus serves as a scaffold to bring TβRII into complex with TβRI-Par6. In this way, Par6 is phosphorylated and cell adhesion at tight junctions, partially regulated through Smurf1 and RhoA, is lost. Inhibition of Par6 phosphorylation has been shown to increase ZO-1-positive mammary epithelial structures and to suppress lung metastasis in an orthotopic mouse model of breast cancer (Viloria-Petit et al., 2009; Viloria-Petit and Wrana, 2010). TGF-β also disrupts baso-lateral adherens junctions via induction of SIP1, a transcription factor capable of transcriptional E-cadherin repression (Comijn et al., 2001). Indirect repression of E-cadherin also occurs through TGF-β induction of other transcription factors, such as Snail1/2, Twist1/2, and ZEB1/2 (Moustakas and Heldin, 2012; Peinado et al., 2003; Thiery et al., 2009).

When TGF-β induces the dissolution of junctional complexes, epithelial cells display altered morphology and behavioral changes. Epithelial cells are thus poised to undergo an epithelial-to-mesenchymal transition (EMT), a state of cellular plasticity in which epithelial cells transiently adopt mesenchymal characteristics. EMT occurs during embryonic development, fibrosis, and cancer (Akhurst, 2008; Taylor et al., 2010; Thiery, 2002; Thiery et al., 2009). TGF-β, one of many pro-migratory factors implicated in EMT, can induce EMT in cultures of normal and transformed cells through various Smad-dependent and -independent pathways, such as the p38, PI3K-Akt, and RhoA axes (Bakin et al., 2000; Bhowmick et al., 2001; Dumont et al., 2003; Levy and Hill, 2005; Saitoh and Miyazawa, 2012). Smads can directly or indirectly activate transcription of mesenchymal markers, including fibronectin, N-cadherin, and α-smooth muscle actin (Moustakas and Heldin, 2012). The full extent to which EMT plays a role in cancer

progression remains highly controversial. With its great similarities to developmental processes, EMT and other types of cell migration, such as collective migration, are undeniable links between development and cancer (Friedl and Gilmour, 2009; Micalizzi et al., 2010). EMT has now been classified into three subtypes to represent distinct biological processes associated with each: Type I EMT is associated with implantation, embryogenesis, and organ development; Type II EMT is in the context of inflammation, tissue regeneration, and fibrosis that occur over extended time periods; and type III EMT enables epithelial invasion and metastasis of transformed cancer cells (Kalluri and Weinberg, 2009; Wendt et al., 2012). Although these subtypes are manifested in unique biological processes, all types of EMT employ a common genetic and biochemical framework for cellular transformation.

Cytostatic regulation, disruption of cell adhesion, and promotion of migration are just a few ways in which autocrine production of and response to TGF-β has been well-studied *in vitro* (Massague, 2008). Collectively, these studies have led to the identification of TGF-β as an early tumor suppressor that switches in function to promote late tumor progression (Inman, 2011). It has been shown that epigenetic downregulation of Dab2, an adaptor protein facilitating R-Smad access to the TGF-β receptor complex, is just one potential factor implicated in the TGF-β functional switch (Hannigan et al., 2010). *In vivo* epithelial cell autonomous TGF-β signaling during tumor initiation, progression, and metastasis has been investigated in several transgenic mouse models (Bierie and Moses, 2006b). Transgenic overexpression of TGF-β1 driven by mouse mammary tumor virus (MMTV) delayed non-metastatic mammary tumor formation in MMTV-TGF-α mice and inhibited tumor formation when these mice were treated with 7,12-dimethylbenz[a]anthracene, a chemical carcinogen (Pierce et al., 1995). TGF-β1 also suppresses benign skin tumor formation (Cui et al., 1996). From a genetic standpoint, one mouse model associated loss of one TGF-β allele with enhanced tumorigenesis in response to carcinogen

treatment (Tang et al., 1998). Although autocrine response to TGF-β1 in these studies is implicated in suppression of tumor formation, it also has been implicated in enhancement of tumor progression and metastasis. TGF-β1 overexpression augmented progression to invasive spindle carcinomas and increased metastatic burden in MMTV-polyoma virus middle T antigen (MMTV-PyVmT) murine mammary tumors (Cui et al., 1996; Muraoka-Cook et al., 2004).

Studies on the roles of TβRI and TβRII in tumorigenesis and progression have offered similar insights as those studies that manipulated TGF-β1 expression. One study coupled constitutively active TβRI with the *c-Neu* transgene and demonstrated enhanced mammary epithelial cell survival and metastasis (Muraoka-Cook et al., 2006). Another study also showed that expression of constitutively active TβRI delayed *c-Neu* tumorigenesis and increased metastasis (Siegel et al., 2003). Consistent with the biphasic dogma regarding TGF-β function, this same study showed dominant negative type II TGF-β receptor (dnTβRII) expression decreased pulmonary metastases in a *c-Neu* breast cancer model. Furthermore, expression of dnTβRII enhanced tumorigenesis in mammary gland, skin, and pancreas mouse models of cancer (Amendt et al., 1998; Bottinger et al., 1997; Gorska et al., 2003; Siegel et al., 2003). In addition to these receptor attenuation models, complete receptor ablation has also been explored using genetically engineered mice with loxP recombination sequences flanking exon 2 of TβRII (Forrester et al., 2005). Cre-mediated recombination at the loxP sites caused mammary tumor-specific ablation of TβRII in MMTV-PyVmT;MMTV-Cre mice. Loss of TβRII decreased tumor latency but enhanced pulmonary metastasis of mammary tumors. The same results were seen using MMTV-PyVMT;WAP-Cre mammary-specific ablation of TβRII (Bierie et al., 2008). Thus, although receptor attenuation and ablation mouse models both identify TGF-β as a tumor suppressor, receptor ablation studies propose a different role of TGF-β during metastasis. Two potential reasons that attenuation and ablation models of TβRII suggest different metastatic

results are the tumor promoters and oncogenes used in the mouse models. Investigation of increased metastasis in the MMTV-PyVmT;MMTV-Cre TβRII ablation model is the subject of our current experimental results presented herein.

Several cell culture and xenograft models have also been used to study epithelial cell autonomous signaling during tumorigenesis and progression. One study demonstrated that TGF-β1 overexpression in a subcutaneous rat prostate cancer model increased both tumor growth and metastasis (Steiner and Barrack, 1992). Another study pinpoints TGF-β1 as a driver of pulmonary metastasis, partially attributed to increased type IV collagenase activity, in a mammary tail vein injection model (Welch et al., 1990). Similarly, splenic injections of the human pancreatic cancer CAPAN-2 cell line, pretreated with TGF-β1, into mice enhanced liver metastasis (Sawada et al., 2006). Use of *in vitro* dnTβRII expression in human MDA-MB-231 breast cancer cells reduced metastatic bone lesions in a cardiac injection model, while expression of constitutively active TβRI in MDA-MB-231 cells enhanced osteolytic bone metastases through the upregulation of PTHrP (Yin et al., 1999).

These *in vivo* models clearly illustrate that overexpression of the TGF-β pathway significantly suppresses tumor formation while it enhances tumor progression and metastasis. Conversely, mouse models also show that attenuation of TGF-β signaling accelerates tumor growth while decreasing metastasis. In contrast, several studies demonstrate that ablation of TGF-β signaling accelerates both tumor growth and metastasis. Studies of TGF-β pathway manipulation thus demonstrate that early tumor suppressive and subsequent pro-tumorigenic effects of TGF-β signaling are cell type- and context-dependent. Much of the autocrine TGF-β responsiveness of epithelial cells can be linked to EMT and invasive and metastatic behavior;

however, paracrine TGF-β secretion and responsiveness in the microenvironment are critical features of dynamic tumor progression and metastasis.

Role of TGF-β in the tumor microenvironment

After numerous studies on epithelial TGF-β signaling, it is now clear that epithelial cell autonomous signaling cannot solely account for TGF-β responsiveness during tumor progression. The mechanisms by which carcinomas lose autocrine growth inhibition but maintain TGF-β-mediated responses during tumor progression have therefore been partially explained by tumor microenvironmental interactions. Epithelial tumor progression relies on paracrine cues emanating from stromal, endothelial, and immune cells in the surrounding milieu (Bierie and Moses, 2006b; Dumont and Arteaga, 2000; Massague, 2008; Taylor et al., 2011). In accordance with the "seed and soil" hypothesis originally proposed by Paget in 1889 to rationalize metastasis, the tumor microenvironment indeed has been shown to foster carcinoma growth through multiple cellular and structural interactions. It is these interactions that make the contribution of TGF-β to carcinoma progression complex and highly context-dependent. In this way, TGF-β is able to mediate cell autonomous, paracrine, and systemic responses that regulate carcinoma progression.

TGF-β signaling causes an accumulation of extracellular matrix (ECM) through the increased synthesis and deposition of ECM components, mainly collagen I and fibronectin, as well as biglycan, decorin, osteopontin, and tenascin; inhibition of matrix-degrading proteases, such as collagenase, stromelysin, and plasminogen activator; and synthesis of matrix-degrading enzyme inhibitors, such as PAI-1 and TIMP-3 (Ignotz and Massague, 1986; Parvani et al., 2011; Schilling et al., 2008). Thus, tumor secretion of TGF-β profoundly remodels the peri-tumoral

stroma, enabling fibroblast recruitment and activation. Fibroblasts also secrete high levels of collagen I and fibronectin, which when combined with stromal TGF-β effects and elevated crosslinking of other ECM components, increase the mechanical rigidity of the tumor microenvironment (Erler and Weaver, 2009). This rigidity helps cells to gain traction employed during migratory behaviors, such as EMT, in addition to increasing contractility and focal adhesions through integrin clustering (Paszek et al., 2005). A stiff extracellular matrix has been associated with malignant transformation and progression (Levental et al., 2009).

Extracellular matrix rigidity has also been associated with myofibroblast expansion and localization at invasive tumor boundaries, implicating myofibroblasts in metastatic progression tumor-stromal interactions (Paszek and Weaver, 2004). TGF-β signaling can stimulate fibroblast transdifferentiation into activated myofibroblasts, which is accompanied by loss of cytokeratin and gain of α-SMA and vimentin expression (De Wever et al., 2008). Myofibroblast transdifferentiation is thus similar to the acquisition of a TGF-β-mediated EMT phenotype, which has been proposed as one potential precursor of myofibroblast presence (Radisky et al., 2007). Using HCT-8/E11 human colon carcinoma cells, it was also shown that carcinoma-secreted TGF-β coordinated the invasion of myofibroblasts through upregulation of myofibroblast N-cadherin expression, dependent upon JNK activity (De Wever et al., 2004b). Additionally, TGF-β-induced transdifferentiation is mediated through αvβ5 and αvβ3 integrins in human mouth and skin fibroblasts and αvβ5 integrin in human kidney fibroblasts (Lygoe et al., 2004). Furthermore, increased expression of fibroblastic αvβ3 clustered and associated with TβRII during TGF-β-stimulated growth of human lung fibroblasts (Scaffidi et al., 2004). These findings suggest an organ-specific relationship between integrin type and responsiveness to TGF-β ligand secretion.

Epithelial-fibroblast interactions are highly reciprocal in that carcinoma-derived TGF-β alters fibroblastic behavior while activated fibroblasts also augment carcinoma progression (Shimoda et al., 2010). The requirement of fibroblast signaling in regulating epithelial growth and tumorigenesis was previously shown in an orthotopic mouse model admixing fibroblasts and ostensibly normal human breast epithelial cells (Kuperwasser et al., 2004). Carcinoma-associated fibroblasts modulate the oncogenic potential of adjacent epithelia, such as breast or prostate, more so than normal fibroblasts (Dakhova et al., 2009; Hayward et al., 2001; Orimo et al., 2005). Myofibroblasts promote epithelial tumorigenesis through secretion of several pro-tumorigenic factors, such as CXCL12 and RhoA- and Rac-dependent tenascin-C and SF/HGF, which increase tumor cell proliferation and invasion (Allinen et al., 2004; De Wever et al., 2004a; Orimo et al., 2005). It is now known that TGF-β is downregulated in fibroblasts of invasive breast cancer tissue as compared to those of normal breast tissue, thereby linking TGF-β signaling in tumor-associated fibroblasts with tumor progression (Singer et al., 2008). Downregulation of TGF-β signaling has been explored in mouse models exploiting conditional deletion of TβRII in fibroblasts via the fibroblast-specific protein 1 (FSP1)-Cre transgene. Conditional TβRII deletion in fibroblasts expands and transforms stromal compartments of the prostate and forestomach resulting in intraepithelial neoplasia and invasive squamous cell carcinoma, respectively (Bhowmick et al., 2004a). In a mammary carcinoma xenograft model, inactivation of TGF-β signaling in mammary fibroblasts exacerbates aggressive tumor behavior, such as growth, scattering, and invasion (Cheng et al., 2005; Cheng et al., 2007, 2008). This occurs through upregulation of TGF-α-, MSP-, and HGF-mediated signaling networks. It has also been shown that loss of one TβRII allele is sufficient to enhance fibroblast accumulation, carcinoma cell invasiveness via CXCL12 and CCL2 cytokines, and pulmonary metastasis in an MMTV-PyVmT mouse model of breast cancer (Fang et al., 2011). These studies all suggest that

paracrine fibroblast TGF-β signaling suppresses the tumorigenic activation of adjacent epithelia. In contrast, the contribution of pancreatic myofibroblasts in advancing pancreatic ductal carcinoma was attributed to Smad-independent TGF-β1-induction of L1CAM in a human pancreatic ductal cancer cell line H6c7 (Geismann et al., 2009). This study suggests that secretion of TGF-β ligand, rather than loss of TGF-β signaling, enhances the tumorigenic potential of adjacent epithelia. Taking together studies of tumor-stromal interactions, it is thus apparent that fibroblasts initially respond to tumor-derived TGF-β early in tumorigenesis but lose this responsiveness during late tumor progression.

In addition to the role of fibroblasts in TGF-β-mediated tumor progression, endothelial cells also facilitate this process. The importance of TGF-β in vascular development has been illustrated in several mouse models of TGF-β signaling and underscores the importance of vascularity in cell maintenance and survival (Derynck et al., 2001). Vascular endothelial cadherin (VE-cadherin) expression and junctional clustering has been shown to increase responsiveness of cells to TGF-β (Rudini et al., 2008). VE-cadherin can physically interact with TGF-β receptors, and its phosphorylation regulates TGF-β-mediated vascular permeability (Shen et al., 2011). Also, the TGF-β disruption of tight junctions via Par6 phosphorylation that occurs in epithelial cells is also present in disruption of endothelial cell adhesion (Townsend et al., 2008). It is therefore not surprising that, similar to the parallels between TGF-β-mediated migration in development and in cancer, TGF-β-mediated regulation of vascularity is also paralleled between development and cancer.

During tumor progression, an intricate vascular network is necessary for nutrient and oxygen receipt by tumor cells, and this network continually expands in response to extrinsic microenvironmental cues during tumor progression. In a chicken allantoic membrane (CAM)

model, TGF-β1 stimulated epithelial and fibroblast accumulation in conjunction with endothelial cell proliferation and angiogenic structure formation (Yang and Moses, 1990). TGF-β stimulates this angiogenesis through both direct and indirect effects. TGF-β-activated myofibroblasts not only modulate epithelial cell behavior through paracrine signaling, but they also stimulate angiogenesis by secreting pro-angiogenic cytokine vascular endothelial growth factor (VEGF) (De Wever and Mareel, 2003). Tumor-secreted TGF-β, just as myofibroblast-secreted TGF-β, upregulates expression of VEGF, which directly stimulates proliferation and migration of endothelial cells (Pertovaara et al., 1994). It has previously been shown that TGF-β expression correlates with VEGF expression in breast and gastric carcinoma patients, and this correlation is associated with poor prognosis for the latter (Donovan et al., 1997; Saito et al., 1999). TGF-β also induces MMP-2 and MMP-9 expression while decreasing TIMP in endothelial cells, thereby enhancing the migratory and invasive properties required by endothelial cells during angiogenesis (Derynck et al., 2001). Indirectly, TGF-β secretion is a chemoattractant for monocytes, which secrete several pro-angiogenic factors, such as FGF, TGF-α, and IGF-1 (Allavena and Mantovani, 2012; Sunderkotter et al., 1991).

In addition to angiogenic stimulation, decreased immune surveillance is an additional significant consequence of TGF-β tumor promotion. This occurs via several mechanisms whereby immune evasion creates a permissive microenvironment for uncontrolled tumor growth (Bierie and Moses, 2010). One mechanism of suppression occurs in lymphoid cells. Attenuation of cytotoxic T lymphocyte (CTL) activity was demonstrated in fibrosarcoma cells transfected with TGF-β1, as well as in EMT6 mammary tumor cells *in vivo* (McAdam et al., 1994; Torre-Amione et al., 1990). Transfection of these EMT6 cells with IL-2 can reverse this effect and induce tumor rejection and reactive CTL activity. Not only does TGF-β facilitate T cell suppression through inhibition of IL-2 production, but it also represses expression of cyclins and

CDK inhibitors, p15, p21, and p27, resulting in decreased T cell proliferation and response (Ahmadzadeh and Rosenberg, 2005; Thomas and Massague, 2005). Additionally, it has been shown that CD4+;CD25+ regulatory T cells work through TGF-β mechanisms to attenuate CTLs (Chen et al., 2005). TGF-β also attenuates the activity of natural killer (NK) cells by downregulating NKp30 and NKG2D receptors required for cytolytic activity (Bellone et al., 1995; Moretta et al., 2000). Similarly, TGF-β attenuates dendritic cell activity by inhibiting dendritic cell maturation and production of proinflammatory cytokines, IL-1 and IL-12, which both contribute to an effective CTL antitumor response (Geissmann et al., 1999).

Recruitment and activity of myeloid cells is also TGF-β-mediated (Yang et al., 2010). TGF-β functions as a chemoattractant for neutrophils and inhibits their ability to recognize and destroy FasL, a ligand expressed on carcinoma cells that promotes cell death (Chen et al., 1998a; Reibman et al., 1991). In contrast to the studies involving TGF-β mediation of immune suppression, it has also been shown that loss of TβRII in mammary carcinoma cells promotes tumor infiltration of Gr-1+, CD11b+ myeloid-derived suppressor cells (MDSCs) (Yang et al., 2008). Increased expression of CXCL1/5 expression by these TβRII-deficient carcinoma cells was associated with the recruitment of tumor-associated macrophages and MDSCs, which are highly immunosuppressive and can inhibit the function of T lymphocytes and NK cells (Bierie et al., 2009; Novitskiy et al., 2011; Taylor et al., 2011). In human cancer, analogous MDSC populations have been observed at the leading invasive edges of breast ductal adenocarcinomas (Yang et al., 2008).

TGF-β-based therapeutic strategies in human cancer

It is known that TGF-β functions in cell type-, context- and temporal-dependent ways to affect tumorigenesis and tumor progression. Implication of TGF-β in the tumor microenvironment is of significant importance in addition to tumor cell autonomous TGF-β signaling. TGF-β-based therapeutic strategies thus become complex since systemic inhibition of TGF-β signaling affects multiple cellular processes and interactions in both homeostatic and carcinoma cells. Due to this inherent complexity, specific targeting of TGF-β-based treatment has been difficult to achieve. Instead, several TGF-β-based therapeutic strategies have been preclinically tested, including large molecule ligand traps, antisense technology, receptor-targeted small molecule inhibitors, and immunomodulatory therapy (Bierie and Moses, 2006b; Korpal and Kang, 2010; Pennison and Pasche, 2007). Timing of treatment is also critical since TGF-β functions as an early tumor suppressor but can subsequently promote tumor progression. In this evolving era of personalized medicine, an understanding of the gene expression profile of an individual patient tumor provides a wealth of information about tumor response to treatment. It is the hope that increased profiling access and accuracy affords better patient stratification into subgroups for tailored cancer therapies.

Overexpression of the ligand, particularly TGF-β1, is predominant in cancer and secreted at a heightened level by both fibroblasts and tumor cells (Bierie and Moses, 2006b; Derynck et al., 1987; Dickson et al., 1987). Inhibition of TGF-β1 in late stages of carcinoma progression can therefore attempt to minimize pro-tumorigenic effects of TGF-β1. Large molecule ligand traps, including monoclonal TGF-β-neutralizing antibodies as well as soluble TβRII and TβRIII proteins, provide means for this inhibition. TGF-β-neutralizing antibodies minimize ligand-receptor interactions, thereby attenuating extracellular TGF-β-mediated

pathway activation. Tested antibodies in cancer include 1D11, 2G7, and GC1008. Both 1D11 and 2G7 mouse neutralizing monoclonal antibodies are pan-TGF-β inhibitors that block the biological activity of all three TGF-β ligands, providing researchers with the ability to mechanistically study the TGF-β pathway. For example, inhibition of TGF-β using 2G7 was found to increase spleen natural killer cell activity, suggesting TGF-β suppression of these cells in tumor progression (Arteaga et al., 1993). Additionally, 1D11 and 2G7 were found to decrease lung metastases in intraperitoneal MDA-MB-231 breast and syngeneic 4T1 mouse models, respectively (Arteaga et al., 1993; Nam et al., 2006). Following this preclinical data GC1008 (Genzyme Corporation), a human pan-TGF-β neutralizing antibody, was tested in a phase I trial for malignant melanoma and renal cell carcinoma patients. Several responders showed reduced liver metastases or reduction in target lesions, lending credence for the current phase II trial expansion (GenzymeOncology, 2012; Morris et al., 2008). A challenge in TGF-β inhibition trials is balancing TGF-β physiological functions in homeostasis and inhibition of excess TGF-β ligand in cancer progression. Thus, one concern of these trials is prolonged TGF-β inhibition and its associated systemic consequences. Despite the fact that TGF-β1-null transgenic mice exhibit an inflammatory response resulting in early death, a study using long-term exposure TGF-β inhibition via 1D11 detected no significant immune dysregulation (Kulkarni et al., 1993; Ruzek et al., 2003). This suggests that neutralizing antibodies may be a viable option for patient treatment.

Truncated, soluble extracellular domains of TβRII and TβRIII proteins (sTβRII and sTβRIII) also function as large molecule ligand traps, just as do TGF-β neutralizing antibodies, by minimizing ligand-receptor interactions. Expression of sTβRII has been found to decrease pulmonary, pancreatic, and liver metastases (Muraoka et al., 2002; Rowland-Goldsmith et al., 2002; Yang et al., 2002). Biogen Fc:TβRII was also shown to increase apoptosis of primary

tumors and to reduce cell motility and intravasation (Muraoka et al., 2002). Similar to chronic exposure of TGF-β neutralizing antibodies, prolonged exposure to sTβRII has not elicited adverse side effects (Yang et al., 2002). The ability of TβRIII to sequester and bind to all three TGF-β ligands, as well as its enhancement of ligand binding to the canonical receptors, identifies sTβRIII as a reasonable means of TGF-β inhibition (Lopez-Casillas et al., 1993). sTβRIII was found to suppress tumor growth and metastasis of MDA-MB-231 breast cancer cells in nude mice, while it also inhibited angiogenesis and growth of HCT116 colon carcinoma and MDA-MB-435 breast carcinoma cell lines (Bandyopadhyay et al., 1999; Bandyopadhyay et al., 2002).

Antisense-mediated TGF-β therapy also targets TGF-β ligand, but rather than inhibit ligand already present in the tumor microenvironment, it interferes with TGF-β synthesis at the mRNA level. In this way, TGF-β secretion into the tumor microenvironment, which is critical in tumor epithelial behavioral response, is reduced. Antisense TGF-β inhibition was effective in reducing tumorigenic and metastatic properties of mouse fibrosarcoma and 4T1 cells in addition to human metastatic breast MDA-MB-435 cells (Moore et al., 2008; Spearman et al., 1994; Wu et al., 2001). Additionally, antisense TGF-β1 inhibition in oncogenic epithelia decreased cell survival, motility, and metastasis in a mammary fat pad mouse model (Muraoka-Cook et al., 2004). This study suggests an autocrine role of TGF-β in cancer progression. An antisense TGF-β1 inhibitor, Antisense Pharma AP11014, was planned for a phase I clinical trial after preclinical studies were successful for human non-small cell lung, colorectal, and prostate cancer cell lines (Schlingensiepen et al., 2004). TGF-β2 has also been targeted using Antisense Pharma AP12009, which reduced cell proliferation and migration of pancreatic and malignant melanoma human cell lines (Schlingensiepen et al., 2006). AP12009 has also been tested in phase I/II clinical trials in recurrent or refractory high-grade glioma patients, resulting in prolonged survival (AntisensePharma, 2012; Hau et al., 2007). Phase IIb/III studies have begun, showing improved

survival in comparison to standard chemotherapy, and phase I/II trials are being conducted in advanced pancreatic carcinoma, metastatic melanoma, and metastatic colorectal carcinoma (AntisensePharma, 2012; Schlingensiepen et al., 2008).

TGF-β receptor-targeted small molecule inhibitors provide yet another avenue by which the TGF-β pathway can be targeted. In contrast to ligand traps or antisense targeting of TGF-β ligands, receptor-targeted small molecule inhibitors block the catalytic activity of the TGF-β kinase receptors by competing for their ATP binding sites. The Scios, Inc. SD208 TβRI kinase inhibitor has been shown to inhibit the growth of murine and human glioma cell lines, as well as inhibiting migration, invasion, primary tumor growth, and pulmonary metastasis of murine mammary cell lines R3T and 4T1 (Ge et al., 2006; Uhl et al., 2004). Another TβRI kinase inhibitor, Kirin Brewery Company Ki26894, decreased motility, invasion, and metastasis of human breast cancer MDA-MB-231 bone tropic cells in addition to prolonging survival (Ehata et al., 2007). GlaxoSmithKline SB-431542, another TβRI kinase inhibitor, also has been shown efficacious in various cell types (Inman et al., 2002). The advent of a TβRI and TβRII dual inhibitor, Eli Lilly & Co. LY2109761, inhibited the metastasis of murine pancreatic carcinoma and murine colorectal carcinoma cells to the liver (Melisi et al., 2008; Zhang et al., 2009). It also reduced tumor growth, enhanced radiation response, and prolonged survival in studies conducted on human glioblastoma multiforme cells (Zhang et al., 2011). Although preventive LY2109761 treatment was efficacious in reducing metastatic burden, it was ineffective at reducing formation of well-developed bone metastases (Korpal et al., 2009). It has also recently been shown that LY2109761 might be most useful for acute administration since it has been reported that long-term exposure to LY2109761 leads to the outgrowth of carcinomas with elevated phosphorylated Smad2 levels that are unresponsive to drug (Connolly et al., 2011). In a clinical setting, Eli Lilly & Co. LY2157299, a TβRI kinase inhibitor, is now in phase Ib/II trials for

recurrent glioblastoma, malignant glioma, hepatocellular carcinoma, and advanced or metastatic pancreatic cancer after exhibiting success in preclinical studies on human Calu6 non-small lung cancer and human MX1 breast cancer cell lines and in a phase I trial (Calvo-Aller et al., 2008; EliLilly, 2012; Yingling, 2012).

Immunomodulatory therapy have targeted TGF-β signaling in immune compartments prior to reconstitution in a tumor-bearing animal, so immunotherapy therefore has not yet reached clinical testing. When TGF-β is secreted by tumor cells into the tumor microenvironment, TGF-β acts as an immune suppressor and renders the immune system incapable of detecting and eliminating carcinoma cells. Since evasion of the immune system therefore contributes to TGF-β-mediated tumor progression, restoration of immune function is important in regaining the ability to suppress tumorigenic activity (Bierie and Moses, 2006b; de Visser and Kast, 1999; Thomas and Massague, 2005). In a transgenic mouse model expressing a dominant-negative TβRII (dnTβRII) in all T cells, murine B16-F10 melanoma and murine EL-4 lymphoma cell lines were unable to metastasize (Gorelik and Flavell, 2001). Another study also demonstrated the importance of TGF-β suppression in T cells using adoptive transfer of dnTβRII-expressing T cells in a mouse prostate cancer model (Zhang et al., 2005). These studies suggest the importance of CD4+, and particularly CD8+, T cell activity in counteracting tumor progression. Immunotherapeutic strategies targeting TGF-β are therefore important in proactively modulating this activity.

Since only a pan-TGF-β neutralizing antibody (Genzyme Corporation GC1008), an antisense TGF-β2 inhibitor (Antisense Pharma AP12009), and a TβRI kinase inhibitor (Eli Lilly & Co. LY2157299) are currently being tested in clinical trials, it begs the question of whether or not TGF-β pathway inhibition would thrive when in combination with other treatments. Preclinical

studies indicate that TGF-β inhibition can indeed augment the therapeutic efficacy of other cytotoxic agents. It has been shown that inhibition of TGF-β1 sensitizes human breast carcinoma cells BT-549 and MDA-MB-468, as well as on human SW-480 colon carcinoma cells, to the apoptotic effects of rapamycin (Gadir et al., 2008). Similar effects were seen with TβRI kinase inhibition in combination with doxorubicin, resulting in reduced tumor growth and lung metastasis in murine 4T1 breast cancer cells (Bandyopadhyay et al., 2010). Another study found that transient TGF-β1 mRNA inhibition enhanced the efficacy of a dendritic cell vaccine by reducing murine B16 melanoma growth and increasing the infiltration of CD4+ and CD8+ T cells (Conroy et al., 2012). All of these preclinical studies suggest that combinatorial treatment utilizing TGF-β inhibition may be promising when moved into the clinical setting.

Summary

Diverse effects of TGF-β signaling are readily observed in homeostasis, development, and cancer processes. TGF-β homeostatic functions include growth arrest and apoptosis. A balance of matrix degradation and deposition is achieved, and TGF-β-initiated migration is used to maintain normal epithelial cell movement during development, regeneration, and wound healing. When epithelial responsiveness to tumor suppressive TGF-β signaling goes awry during tumorigenesis and progression, autocrine TGF-β signaling becomes a dominant factor. TGF-β switches to a tumor-promoting role and can facilitate disruption of cell junctional complexes retained in untransformed cells. Dissolution of adhesion complexes is one initiator of an EMT-phenotype frequently associated with enhanced motility and invasion. Signaling is also associated with increased metastatic behavior both in *in vivo* models and in clinical cancer samples.

During tumor progression, high levels of TGF-β ligand secretion present in the tumor microenvironment are concomitant with a decrease in epithelial TGF-β responsiveness. Microenvironmental cells therefore respond to the abundant amount of TGF-β readily available in the tumor milieu. Thus, TGF-β-mediated paracrine interactions are crucial in aiding tumor progression. Mechanical rigidity of the extracellular matrix enhances the migratory ability of both epithelial cells and adjacent stromal cells, lending a permissive environment for cell transformation and tumor growth. Activated myofibroblasts surround the primary tumor and can modulate the oncogenic potential of adjacent epithelia, such as stimulation of invasion and proliferation. Stromal endothelial cells are also involved in tumor angiogenesis via TGF-β-mediated direct and indirect effects. In addition, TGF-β-mediated attenuation of immune surveillance further potentiates oncogenic transformation. Suppression of both CTL and NK cells reduce tumor rejection and facilitate tumor growth and progression. Also in a pro-tumorigenic fashion, TGF-β is capable of neutrophil and MDSC recruitment, exacerbating tumor inflammation, growth, and metastasis.

The plasticity of tumor responsiveness is therefore a consequence of diverse ligand, receptor, and cell type levels. The net effect of TGF-β signaling in the microenvironment is thus a complex tumor network with multiple inputs and outputs acting coordinately to suppress or promote tumor progression. Deciphering the biphasic effects of TGF-β signaling in multiple cell types, and coordinately, is critical to understanding the expansive role of this signaling pathway in cancer progression. It has previously been shown that loss of TβRII in mammary carcinoma cells potentiates pulmonary metastasis five-fold (Bierie et al., 2008; Forrester et al., 2005). Although attributed to enriched macrophage populations and cytokine secretion, this increased metastasis was not investigated in the context of tumor-stromal interactions and cell migration. In our studies presented herein, we have investigated both tumor-stromal interactions and

temporal contributions of TGF-β signaling during tumor progression. Our results demonstrate that tumor-stromal interactions are required for carcinoma cell migration and invasion. Furthermore, these interactions promote enhanced extravasation and proliferative ability at secondary colonization sites of metastasis. At these sites, we have shown that re-epithelialization of carcinoma cells occurs. We have linked enhanced migration and metastasis to loss of TGF-β signaling in carcinoma cells, facilitating the formation of collectively migrating aggregates. We have therefore identified TGF-β as a critical modulator of single cell versus collective cell migration. Associated gene expression data for collective migration was obtained, revealing downregulation of Tmeff1 and other Nodal signaling inhibitors. Additionally, we have explored the timing of TGF-β signaling during mammary tumor progression in an *in vivo* model. Other systems have used either constitutive conditional ablation or constitutive dominant-negative expression of TβRII. We have utilized an inducible expression system for dominant-negative TβRII that supports the tumor suppressive role of TGF-β. Inhibition of TGF-β prior or at tumor onset significantly increased pulmonary metastasis. Taken together, our results suggest that loss of carcinoma TGF-β signaling enhances migratory and metastatic properties of tumor epithelia.

CHAPTER II

TGF-β SIGNALING REGULATION OF MAMMARY CARCINOMA CELL INVASION DURING TUMOR-STROMAL CROSSTALK

Introduction

Transforming growth factor-beta (TGF-β) is a pleiotropic cytokine that regulates growth arrest, cell motility, development, and differentiation (Akhurst and Derynck, 2001; Bierie and Moses, 2006a, b; Derynck et al., 2001). TGF-β signaling is also instrumental in the tumor microenvironment by influencing both tumor development and metastasis (Bierie and Moses, 2006b), and it is frequently dysregulated in breast cancers (Gobbi et al., 2000; Hinshelwood et al., 2007; Levy and Hill, 2006). In the mammary epithelium, attenuation of TGF-β signaling using a dominant negative type II TGF-β receptor (dnTβRII) resulted in lobular alveolar hyperplasia and an increased rate of tumor formation in conjunction with a *TGF-α* transgene (Gorska et al., 2003); however, decreased pulmonary metastasis resulted when dnTβRII was expressed along with a *c-Neu* transgene (Gorska et al., 2003; Siegel et al., 2003). Conversely, activation or overexpression of TGF-β signaling in mammary carcinoma cells expressing either the *c-Neu* transgene or polyoma virus middle T antigen (PyVmT) transgene delayed tumor onset but enhanced pulmonary metastasis (Muraoka-Cook et al., 2004; Muraoka-Cook et al., 2006; Siegel et al., 2003). Taken together, these observations suggest a tumor suppressive role of TGF-β during tumor initiation and early tumor progression, while additionally implicating TGF-β in promotion of late-stage tumorigenesis. Mammary-specific ablation of the type II TGF-β receptor (TβRII) also supported the role of TGF-β as a tumor suppressor but challenged the dogma of TGF-β as a metastatic promoter. Conditional knockout of TβRII in mammary epithelial cells

expressing PyVmT led to decreased tumor latency; however, in contrast to attenuated TGF-β signaling models, TβRII ablation increased pulmonary metastasis (Bierie et al., 2008; Forrester et al., 2005).

This dual role of TGF-β as both tumor suppressor and promoter has therefore presented a dichotomy in which TGF-β signaling is context- and cancer type-dependent. Consequently, epithelial-autonomous TGF-β signaling cannot solely be responsible for influencing tumor behavior. The tumor microenvironment, an abundant source of TGF-β (Bierie and Moses, 2006b), is comprised of diverse cell populations, such as epithelial, stromal, vascular, and immune cells, working coordinately to promote tumor progression. Epithelial-stromal crosstalk in tumorigenesis has garnered much attention. It has been shown that epithelial TGF-β signaling regulates fibroblast recruitment and activation (Bhowmick et al., 2004b; Bierie and Moses, 2006b). Concurrently, stromal TGF-β signaling suppresses tumorigenesis in adjacent epithelia while its ablation potentiates tumor formation (Bhowmick et al., 2004a; Cheng et al., 2005). Fibroblasts can also lead carcinoma cells along self-generated extracellular matrix tracks during carcinoma cell migration and invasion (Gaggioli et al., 2007). Transient TGF-β signaling in these invading cells can induce single motility, permitting hematogenous and lymphatic invasion (Giampieri et al., 2009b; Matise et al., 2009). In contrast, lack of active TGF-β signaling results in collective invasion and lymphatic spread (Giampieri et al., 2009b). This illustrates the important role of carcinoma cell TGF-β signaling in determining the mode of cell migration and invasion.

The adaptability of invading cells is evident in multiple forms of cell migration. Single cells invade in either an amoeboid or mesenchymal manner characterized by non-epithelial morphology, loss of cell-cell contacts, and presence of actin stress fibers (Friedl and Wolf, 2003). Whereas amoeboid cells move through matrix pores, mesenchymal migration

additionally employs proteolytic remodeling of the extracellular matrix. Collective invasion also relies on local remodeling of the extracellular matrix (Wolf et al., 2007) and occurs by two-dimensional sheet migration or three-dimensional group or strand migration (Friedl and Gilmour, 2009). These cellular cohorts are heterogeneous, comprised of leading and following cells. Leading cells, which may exemplify mesenchymal properties, survey microenvironmental surroundings, relay extrinsic guidance cues to following cells, and forge clustered migration (Revenu and Gilmour, 2009). In breast cancer, amoeboid, mesenchymal-like, and collective cell migration have all been identified (van Zijl et al., 2011). Inflammatory breast cancer, associated with high rates of metastasis and mortality, is marked by evidence of tumor emboli or clusters that maintain p120 and E-cadherin expression through translational control (Silvera et al., 2009). Collective clusters are also characteristic of invasive ductal carcinoma (Florentine B, 1999). On the other hand, lobular carcinoma frequently manifests single cell or strand migration (Bierie and Moses, 2006a; Tarin et al., 2005).

It is well-established that TGF-β potently stimulates cellular migration and invasion. TGF-β induces epithelial and fibroblast migration, promotes fibroblast transdifferentiation into invasive myofibroblasts, and drives an epithelial-to-mesenchymal transition (EMT) frequently associated with invasive tumors (Bhowmick et al., 2001; Bierie and Moses, 2006a; Yang and Moses, 1990; Zavadil and Bottinger, 2005). These observations support the hypothesis that TGF-β regulates migration patterning through tumor microenvironmental interactions, such as epithelial-stromal crosstalk. These spatially, temporally, and biologically complex interactions can make *in vivo* TGF-β signaling studies difficult. We therefore chose to study epithelial-stromal crosstalk through an integrated systems analysis, combining genetically engineered mouse models and the use of the chicken embryo chorioallantoic membrane (CAM) model (Zijlstra et al., 2008). Mammary tumor cells xenografted onto the CAM thrive in large part due

to robust vascularization of the nascent tumor in the CAM. The CAM model also offers several advantages over other model systems. First, the *ex ovo* model affords long-term intravital imaging for up to 72 hours of continual imaging. Second, this model system enables real-time tracking of cellular behavior throughout the embryo lifespan, allowing for multiple imaging timepoints without compromising host viability. Lastly, in both the *ex ovo* and *in ovo* models, the chicken embryo presents minimal xenograft rejection since the embryo maintains immature, maternal B cell populations incapable of full immune activity (Eskola, 1977; Stern, 2005). Using both the *ex ovo* and *in ovo* CAM models, we characterized how tumor cell migration and invasion utilizes TGF-β-mediated epithelial-stromal interactions. We found that mammary fibroblasts enhance the migratory potential of carcinoma cells in either a single cell/strand migration when epithelial TGF-β signaling is present or a collective migration in its absence. Furthermore, the collective migration and invasion observed correlated with increased metastasis. Our data demonstrates that carcinoma cell TGF-β signaling regulates migration patterning, metastasis, and junctional protein expression at the invasive tumor front. It also implicates a TGF-β-mediated cell-autonomous migratory behavior evident only during stromal influence on epithelial cells.

Experimental Procedures

Cell lines, transfection, and treatment

Mammary tumor epithelial cells, isolated from either mouse mammary tumor virus (MMTV)-PyVmT; MMTV-Cre; $T\beta RII^{fl/fl}$ (TβRII KO) or MMTV-PyVmT; $T\beta RII^{fl/fl}$ (control) mice (Forrester et al., 2005), and Fsp-Cre; $T\beta RII^{fl/fl}$ (partial TβRII KO) fibroblasts (Bhowmick et al., 2004a) were used in xenografts for *ex ovo* and *in ovo* CAM assays. Both types of epithelial cells were transduced with

lentiviral EGFP (kind gift from the Pietenpol laboratory, Vanderbilt University) for intravital imaging. Fibroblasts were labeled with a cell permeable dye ($DiIC_{18}$(5)-DS, Molecular Probes). For all cell combination experiments, fibroblasts were used at a 2.5:1 ratio to promote the most aggressive behavior of epithelial cells (data not shown). A human TβRII retroviral construct (Addgene plasmid 19147) was used for reconstitution of TGF-β signaling in TβRII KO epithelia. Phoenix packaging cells were transfected with 8 μg of construct for 6 hours, followed by 48 hour viral production. TβRII KO epithelia were then infected for 6 hours and subsequently maintained with 1 μg/mL puromycin for selection. Additionally, any TGF-β treatment of cell lines was completed using 1 ng/mL TGF-β1 (R&D Systems) for 2.5 hours prior to RNA or protein collection.

Transwell invasion assay

Matrigel™ (BD Biosciences) was diluted 1:10 in PBS and coated on the bottom of 8 μm pore Transwell® filters (Corning Incorporated) for 30 minutes at 37°C. For conditions in which both epithelial cells and fibroblasts were used, fibroblasts (100,000) were then immediately seeded onto the diluted Matrigel™-coated filter and incubated for 15 minutes at 37°C. All transwell filters were placed upright in a 24 well plate in DMEM/10% FBS and incubated overnight at 37°C. On the following day, $T\beta RII^{fl/fl}$ or TβRII KO epithelial cells (40,000) were seeded on the top of the coated transwell filter in serum-free DMEM. In conditions in which fibroblasts were used, the number of fibroblasts used was therefore at a 2.5:1 ratio compared to that of epithelial cells. Transwell filters were incubated at 37°C for the entire duration of the 6 hour invasion assay. Filters were fixed in 10% NBF, washed, and dried overnight. Invaded cells were visualized on the filters using ProLong® Gold antifade reagent with DAPI nuclear stain (Invitrogen™).

***Ex ovo* CAM assay**

Chicken embryos were placed into sterile weigh boats with plastic lids at day 4 post-incubation. On day 10 post-incubation, EGFP-expressing mammary epithelial cells alone or in combination with fibroblasts were grafted onto the CAM. Intravital imaging began on day 12 post-incubation. Fully-automated upright fluorescent microscopes (Olympus BX61 WI and BX60 M) were used for imaging fluorescent cells. Time-lapse images were captured every 15 minutes for the duration of the experiment. Analysis of cell velocity, migration distance, and digital processing was achieved through Volocity® software (Improvision) using protocols previously described (Zijlstra et al., 2008). Two-photon microscopy of CAM tumors was subsequently completed (Vanderbilt Cell Imaging Shared Resource). Embryonated eggs for all chicken CAM assays were graciously provided by the Tyson Food Corporation.

***In ovo* CAM assay**

The CAM was prepared as previously described (Palmer et al., 2011). Briefly, the CAM was dropped from the eggshell on day 10 post-incubation. At this time, mammary epithelial cells alone or in combination with fibroblasts were grafted onto the CAM. Tumor-bearing animals were sacrificed and tumor tissue and distant CAM were collected 7-10 days post-grafting. Distant CAM was classified as any part of the CAM in which the primary tumor was not grafted. In this way, any piece of distant CAM is a metastatic site. In order to collect distant CAM at the time of sacrifice, the eggshell was cut radially into two equivalent halves. Two circular areas of CAM, identical in size, were harvested from each half using a boring tool. The resulting four pieces of CAM were then analyzed via murine *Alu* PCR for the presence of disseminated cells. In conjunction with quantitative analysis of distant CAM, blood was also collected from tumor-bearing chicken embryos 10 days post-grafting of both epithelial cells and fibroblasts to analyze

cells in circulation. RNA isolation was subsequently isolated from the collected blood using a Mouse RiboPure™-Blood RNA Isolation Kit (Ambion®) followed by a DNA-*free*™ kit (Invitrogen™). qPCR quantification of intravasated cell RNA was found via PyVmT and EpCAM expression (primer sequences found in "Expression analysis" section below). C_t values were subjected to statistical analyses after normalization to chicken β-actin (Grommen et al., 2006).

Murine *Alu* PCR

In order to quantify metastatic cell dissemination in the CAM, CAM DNA was first extracted using the SYBR® Green Extract-N-Amp Tissue PCR Kit (Sigma). DNA was then analyzed through the use of quantitative murine *Alu* PCR (5' GGGCTGGTGAGATGGCTCAGTGG 3' forward, 5' CTTCAGACACACCAGAAGAGGG reverse) (Zijlstra et al., 2002). C_t values were subjected to statistical analyses after normalization to chicken GAPDH (5' GAGGAAAGGTCGCCTGGTGGATCG 3' forward, 5' GGTGAGGACAAGCAGTGAGGAACG 3' reverse).

***In ovo* experimental metastasis assay**

Injections were performed as previously described (Palmer et al., 2011). In brief, fluorescently labeled carcinoma cells alone or in combination with fibroblasts were injected intravenously into the allantoic vein of the embryo on day 12 post-incubation. Initial cell arrest was assessed at 6 hours, and subsequent extravasation and proliferative capability was assessed at 18 and 24 hours (72 hours was used as an additional time point for Figure 2D). At these time points, cell dissemination was analyzed as described above (*In ovo* CAM assay). In order to label the host chicken vasculature, embryos were injected intravenously with 100 µl of 500 µg/mL rhodamine *lens culinaris* agglutinin (Vector Laboratories) into the allantoic vein. Imaging of epithelial cells and host vasculature was completed using a fully-automated upright fluorescent microscope (Olympus BX61 WI). Digital processing was achieved through Volocity® software (Improvision).

Laser capture microdissection and expression analysis

Laser Capture Microdissection (LCM) was performed on 5 µm frozen *in ovo* tumor sections on an Arcturus PixCell IIe microscope at the Vanderbilt Translational Pathology Shared Resource. LCM-captured RNA was isolated using an RNAqueous-Micro kit (Ambion) and validated for array quality (Vanderbilt Genome Sciences Resource). Subsequent cDNA synthesis and amplification was completed using a RT^2 Nano PreAMP cDNA Synthesis Kit (SA Biosciences). Samples, 3 control tumors and 3 KO tumors, were individually assayed on EMT RT^2 Profiler™ qPCR Arrays (SA Biosciences) in a BioRad iCycler. Analysis was completed using web-based RT^2 Profiler™ PCR Array Data Analysis (SA Biosciences). Selected gene targets were either 10-fold or greater upregulated or downregulated when comparing our TβRII KO tumors to our $T\beta RII^{fl/fl}$ tumors.

Expression analysis

Total cell RNA was collected using TRIzol (Invitrogen) and further purified using an RNeasy Mini Kit with RNase-Free DNase (both Qiagen). cDNA was synthesized using either Superscript III reverse transcriptase (Invitrogen) or SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) as described by the manufacturer. Bio-Rad iCycler and CFX96 machines were used for qPCR employing *Power* SYBR® Green (Applied Biosystems) or SsoAdvanced SYBR® Green Supermix (Bio-Rad), respectively. The following primer sequences were used to amplify murine coding sequences of interest: FAP (5’ CCAGGAGATCCACCTTTTCA 3’ forward, 5’ GTGGCAAGCATTTCCTCTTC 3’ reverse), PyVmT (5’ TAAGAAGGCTACATGCGGATGGGT 3’ forward, 5’ GGCACCTGGCATCACATTTGTCTT 3’ reverse), EpCAM (5’ AAGCCCGAAGGGGCGATCCA 3' forward, 5' GTGCCGTTGCACTGCTTGGC 3' reverse), Wnt11 (5' TCCTGGGCTGGCAGGAGGAC 3' forward, 5' GACCAGGTCGGAGGACCGGG 3' reverse), Tmeff1 (5' GCCGAGTGTGACGAGGATGCG 3' forward, 5' AACTCCCGTCGGAAGCGCAC 3' reverse), Dsc2 (5' GCCCAGAGCTCCACCCTCGGA 3'

forward, 5' ACACAGGCGCTTTTCTCGCGC 3' reverse), Snai3 (5' CCACACGCTGCCCTGCATCT 3' forward, 5' GGGTGCGGATGTGACCCTGG 3' reverse), eIF4GI (5' CCGGTGGTGTTTAGCACGCCTC 3' forward, 5' CGGCTAGGGTAGAAGTGCTGCAG 3' reverse), DDR1 (5' GCCATGGTCACCTTGAAGCCAGC 3' forward, 5' CGATGAAGCCTCCCGGCTTTGTC 3' reverse), Tmeff1 follistatin domain 1 (5' ATGCGCATGCCAGTTTCAGTGCC 3' forward, 5' GCAAGCAGCCCTCCTGAGAAAGC 3' reverse), Tmeff1 EGF domain (5' GGGTACTGCATCCATGGGAAATGTG 3' forward, 5' CCGTCTTCTCACAGTGCTGTCCA 3' reverse), Tmeff1 transmembrane/cytoplasmic domains (5' CGCAGCCATTATTGGAGCAGTACA 3' forward, 5' CCGCCCTCTATTGTTCTTGGGGC 3'), Tmeff1 cytoplasmic domain (5' CATGTGCATAACAAGGAAATGCCCC 3' forward, 5' AAAATGACCCAGGTTCTGCTTCTGC 3' reverse), Dact2 (5' GGAGATGTGGGCACCGAGCG 3' forward, 5' GGCCAGTGCGGCTCGTAGTC 3' reverse), SnoN (5' GGCCACCAAGGCAGAGACAAATTC 3' forward, 5' GCTTGTGCCTCTCACTAAGCTGC 3' reverse), Gsc (5' CGCCGAGCCAAGTGGAGACG 3' forward, 5' CCGGCGAGGCTTTTGAGGACG 3' reverse), Mixl1 (5' CGCAAGCGCACGTCGTTCAG 3' forward, 5' GCGCTCCCGCAAGTGGATGT 3' reverse), and GAPDH (5' AGAACATCATCCCTGCATCC 3' forward, 5' CACATTGGGGGTAGGAACAC 3' reverse). C_t values were subjected to statistical analyses after normalization to GAPDH.

Immunohistochemistry and immunofluorescence

In ovo tumors were harvested, fixed in 10% NBF, and then paraffin-embedded and sectioned (Vanderbilt Translational Pathology Shared Resource). H&E sections were used to identify host (chicken) immune infiltrates for histological evaluation for a total of 5 tumor sections per tumor type ($T\beta RII^{fl/fl}$ or TβRII KO) and 2 fields per infiltrate. Infiltrating monocytes and heterophils were identified by nuclear morphology, cytoplasmic coloring, and morphometry and then subsequently counted. Monocytes were identified by their mononuclear morphology, while

heterophils were distinguished by their variably lobulated nucleus. All immunohistochemistry and immunofluorescence on *in ovo* tumor sections involved blocking via incubation with 3% normal goat serum (Vector Laboratories). Immunohistochemistry for E-cadherin and phospho-Smad2 was completed by the Vanderbilt Translational Pathology Shared Resource. All immunofluorescence was done using a standard pH 6 sodium citrate buffer. Immunofluorescence data was obtained using primary antibodies for vimentin (1:500; Covance PCK-594P), α-SMA (1:500; Sigma A2547), E-cadherin (1:500; BD Transduction Laboratories 610181), cytokeratin 8/18 (1:500; Fitzgerald 20R-CP004), ZO-1 (1:500; Zymed 61-7300), p120 (1:400; BD Transduction Laboratories 610133), and β-catenin (1:1000; Sigma C2206) by incubation overnight at 4°C. Corresponding Alexa Fluor® secondary antibodies were used (1:1000; Invitrogen). Fluorescent imaging was completed on a Zeiss Axioplan upright widefield microscope.

Immunoblotting

Protein lysate preparation and immunoblotting procedures were used as previously described (Bierie et al., 2008). PVDF membranes were blocked in 5% milk in TBST and incubated with primary antibody overnight at 4°C. The following primary antibodies were used: phospho-Smad2 (1:1000; Millipore AB3849), TβRII (1:4000; Santa Cruz Biotechnology, Inc. sc-400), Wnt11 (1:1000; Abcam ab96730), Tmeff1 (1:1000; Santa Cruz Biotechnology, Inc. sc-98956), Versican (1:1000; Millipore AB1033), and N-cadherin (1:2500; BD Transduction Laboratories 610920). Corresponding secondary HRP ImmunoPure® antibodies were used (1:5000; Pierce). Chemiluminescence detection of protein was completed using Western Lightning® ECL (PerkinElmer).

Statistics

All statistical analyses were reported using two-tailed unpaired *t* tests to determine significance ($p<0.05$).

Results

Fibroblasts induce single cell/strand or collective migration of epithelia

In order to assess the inherent migratory differences between our murine MMTV-PyVmT TβRII KO or MMTV-PyVmT TβRII$^{fl/fl}$ control mammary carcinoma cells, the use of an *ex ovo* chicken embryo model system was employed. Initial grafting was of EGFP-expressing murine MMTV-PyVmT mammary tumor epithelial cells, either TβRII KO or TβRII$^{fl/fl}$ alone, which were allowed to form discernible, vascularized tumors for three days. Tumor-bearing animals were placed in an intravital imaging chamber and tumor cell motility was evaluated for up to 72 hours via time-lapse imaging. We observed a consistently larger tumor size of TβRII KO tumors compared to TβRII$^{fl/fl}$ control tumors; however, both tumors presented no evidence of migration beyond the periphery of the primary tumor (**Figure 1A**). The lack of an inherent difference in migratory activity due to the presence or absence of TGF-β signaling in the epithelial cells confirmed that the previously published elevated lung metastasis observed in our TβRII KO mice was not due to enhanced cell-autonomous migratory capacity of TβRII KO epithelial cells alone. We therefore hypothesized that stromal influence on epithelial cells could critically alter the migration pattern of tumor epithelial cells.

In order to best recapitulate tumor-stromal interactions of the tumor microenvironment, the TβRII$^{fl/fl}$ and TβRII KO epithelial cells were combined with partial TβRII KO

mammary fibroblasts *ex ovo* (hereafter, fibroblasts are grafted with epithelial cells in all tumors). Partial TβRII KO fibroblasts were used due to their ability to invoke more aggressive tumor behavior as compared to that of pure TβRII KO fibroblasts or TβRII competent fibroblasts (Cheng et al., 2005). For all *in vivo* experimentation, unless otherwise noted, only partial TβRII KO mammary fibroblasts were used. In both $T\beta RII^{fl/fl}$ and TβRII KO tumors, the presence of fibroblasts caused epithelial migration away from the tumor periphery (**Figures 1A, 1B**). In control $T\beta RII^{fl/fl}$ tumors capable of TGF-β signaling, the tumor cells exhibited a strand and/or single cell migration (**Figures 1B, 2A**). Notably, collective migration was not observed in any $T\beta RII^{fl/fl}$ tumors. In contrast, TβRII KO tumors exhibited primarily collective migration with occasional single cell or strand migration (**Figures 1B, 2A**). In either tumor type, fibroblasts were always visible outside of the tumor mass beyond the periphery of invading tumor cells, reaffirming the concept that stromal cells lead the way for subsequent tumor cell migration. This corroborates *in vitro* data indicating that fibroblasts enhanced the invasion of epithelial cells in a transwell assay (**Figure 2B**). The two migratory phenotypes observed *in vivo* were also affected by vascular influence in the tumor microenvironment. Migration appeared directional, as epithelial cells migrated along and around the vasculature (**Figure 2C**), perhaps due to migratory cues emanating from the vasculature or characteristics of the perivascular matrix.

Since the fibroblasts had a pronounced effect on tumor cell migration, a reciprocal effect of tumor cell influence on fibroblasts was investigated. No difference in displacement rate of fibroblasts from the tumor periphery was observed regardless of their combination with either $T\beta RII^{fl/fl}$ or TβRII KO carcinoma cells; however, fibroblast velocity was increased by 50% in the presence of TβRII KO cells (**Figure 2D**). In this way, the TβRII KO epithelial cells, which possess an increased propensity for lung metastasis (Bierie et al., 2008; Forrester et al., 2005),

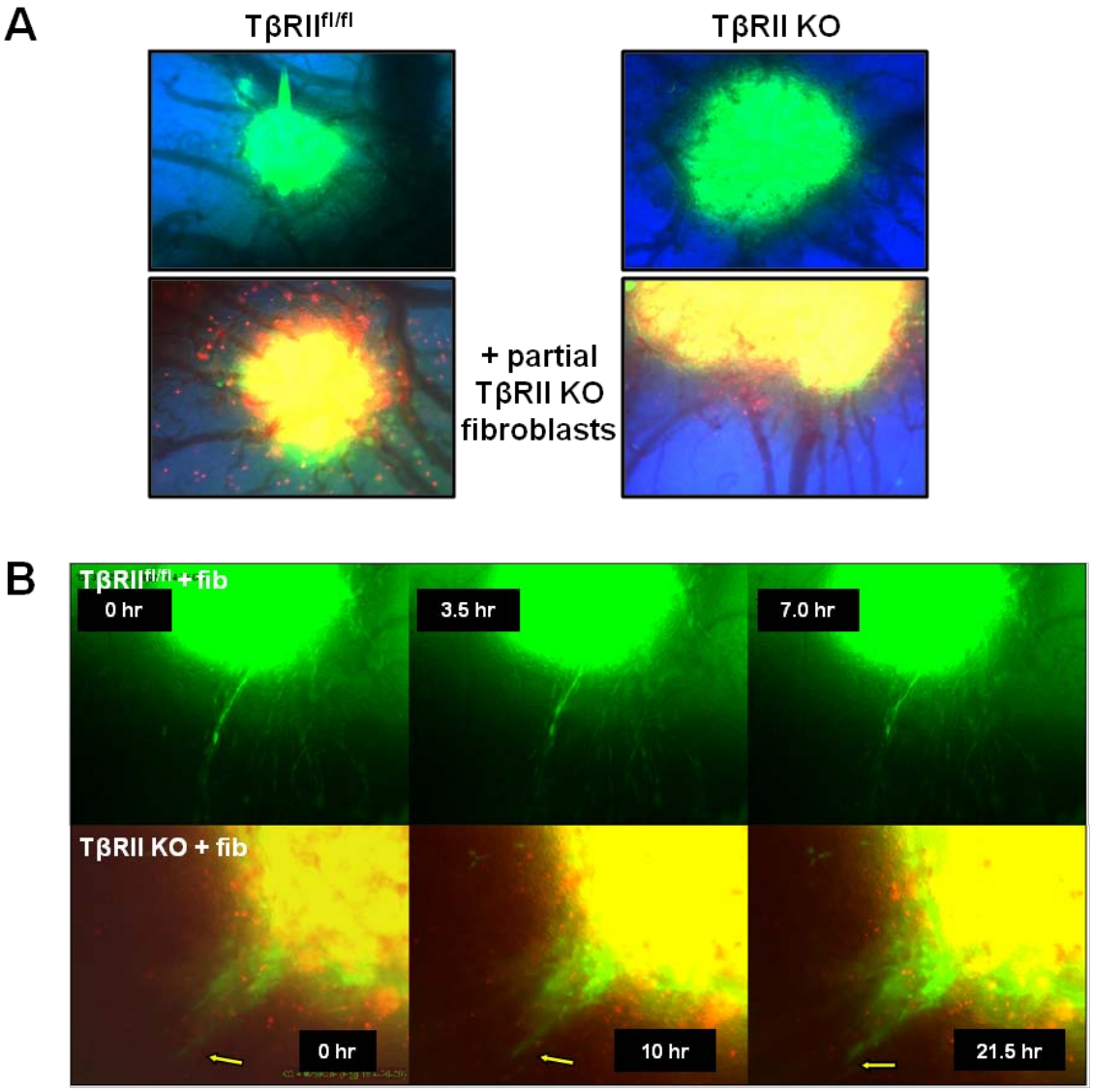

Figure 1. Fibroblasts invoked tumor growth and migratory behavior of carcinoma cells.

(A) Fibroblasts caused increased tumor growth of both TβRII$^{fl/fl}$ and TβRII KO tumors (top panels are epithelial cells alone; bottom panels are epithelia and fibroblasts combined). Epithelial cells are seen in green, and fibroblasts are overlayed in red. **(B)** Combinatorial xenografts of either EGFP-labeled TβRII$^{fl/fl}$ control or TβRII KO carcinoma cells with fibroblasts were put onto the CAM and monitored via intravital imaging. In the top panel, single cell migration was exhibited in tumors that maintain epithelial TGF-β signaling. Only the epithelial channel is shown in order to visualize the single cell and strands displayed. In the bottom panel, collective migration was observed in TβRII KO tumors (*arrow*). Both epithelial (green) and fibroblast (red) channels are overlayed. Fibroblasts guided both types of epithelial migration.

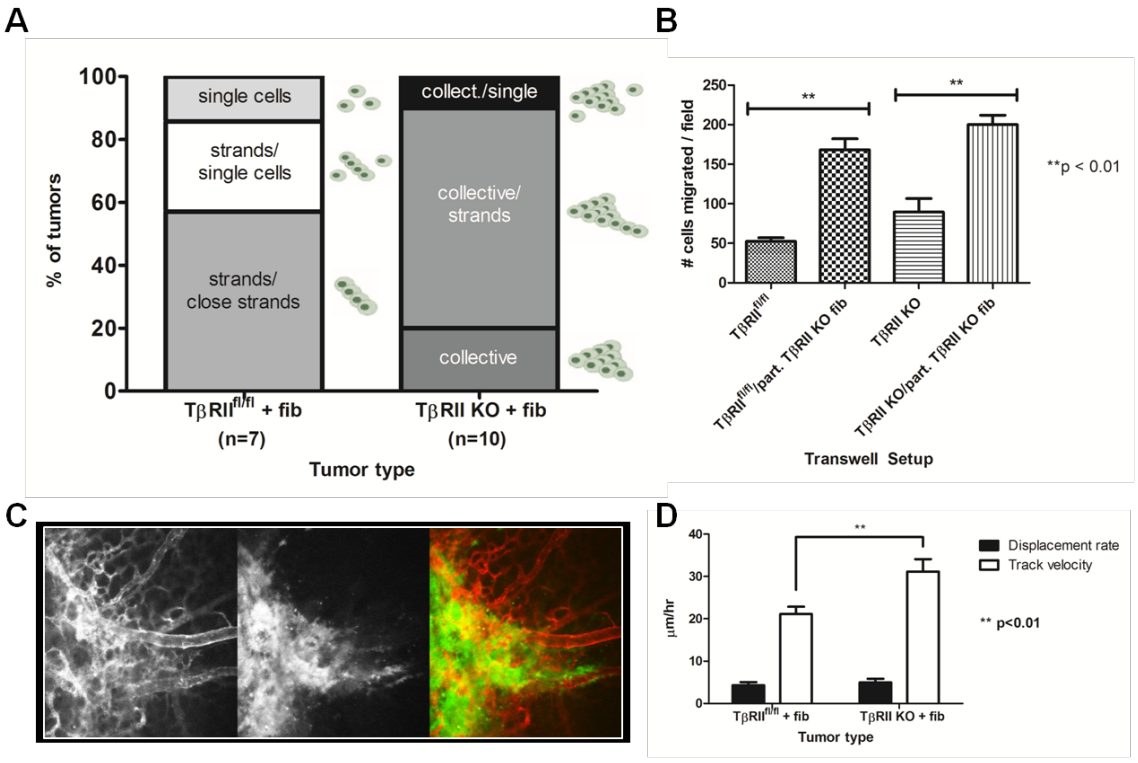

Figure 2. Tumor-stromal interactions promoted either single cell or collective cell invasion.

Combinatorial xenografts of either EGFP-labeled TβRII$^{fl/fl}$ control or TβRII KO carcinoma cells with fibroblasts were put onto the CAM and monitored via intravital imaging. **(A)** The graph quantifies migration types observed when comparing TβRII$^{fl/fl}$ control and TβRII KO *ex ovo* tumors. **(B)** Fibroblasts enhanced invasion of carcinoma cells through Matrigel™-coated transwells after 6 hours. Carcinoma cells were permitted to invade through Matrigel™ alone. Carcinoma cells were also allowed to invade through Matrigel™ that had a bottom fibroblast coating; this condition was used to assess tumor-stromal interactions. **(C)** TβRII KO tumors migrated collectively along and around the vasculature, as shown by two-photon microscopy. Vasculature (left), epithelial (middle), and overlayed (right) panels are shown. **(D)** Fibroblasts had enhanced velocity in the presence of TβRII KO epithelial cells compared to TβRII$^{fl/fl}$ cells.

responded to extrinsic stromal cues in a heightened manner and subsequently facilitated tumor-stromal communication. This reciprocity of tumor-stromal interactions in driving motility and invasion is consistent with previously observed interactions in the tumor microenvironment of other models (Bhowmick et al., 2004a; Bhowmick et al., 2004b; Bierie and Moses, 2006b; Hanahan and Weinberg, 2011).

Although partial TβRII KO fibroblasts were used in all experiments due to their ability to invoke more aggressive tumor behavior as compared to that of pure TβRII KO fibroblasts or TβRII competent fibroblasts (Cheng et al., 2005), each of these fibroblast cell lines was also tested in our chicken embryo model for effects on migration patterning. Either $T\beta RII^{fl/fl}$ or TβRII KO carcinoma cells combined with each of these three fibroblast types were xenografted onto the CAM *in ovo*. These combinatorial tumors were significantly larger, as compared to controls, when using TβRII KO carcinoma cells combined with either TβRII competent or partial TβRII KO fibroblasts (**Figure 3A**). Furthermore, histological comparison between the tumor types revealed single cell and collective phenotypes for all $T\beta RII^{fl/fl}$ and TβRII KO tumors, respectively (**Figures 3B, 3C**). The consistency of these migratory phenotypes suggests that the presence of active TGF-β signaling in fibroblasts does not affect epithelial migration patterning.

Cell migration mode can affect metastatic potential

Histological evaluation of fixed tumor tissue was used to determine cellular morphology within the tumor. For this purpose, mammary carcinoma cells, either $T\beta RII^{fl/fl}$ or TβRII KO, were combined with mammary fibroblasts and xenografted onto the CAM *in ovo*. Overall tumor histology revealed a well-differentiated, lobular morphology in $T\beta RII^{fl/fl}$ control tumors; however, the TβRII KO tumors appeared less differentiated (**Figure 4A**). The tumor histology is

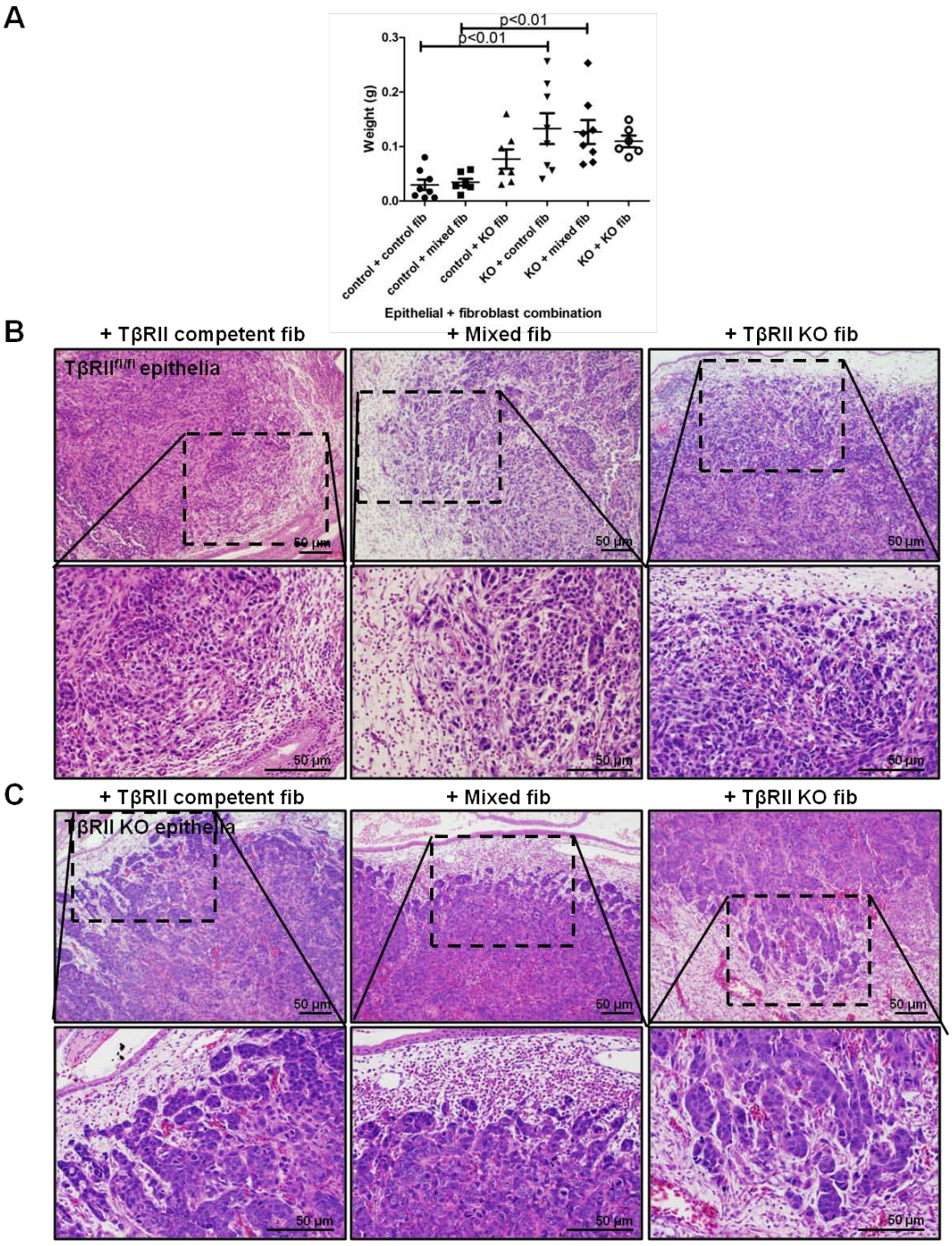

Figure 3. Fibroblasts, regardless of active TGF-β signaling, induced either single cell or clustered epithelial migration.

(A) Control epithelia (TβRII$^{fl/fl}$) and TβRII KO epithelia were combined *in ovo* with TβRII-competent fibroblasts, TβRII KO fibroblasts, or a 50/50 mixture of these fibroblasts. Tumor weights are shown. H&E sections of all TβRII$^{fl/fl}$ and TβRII KO tumors are seen in **(B)** and **(C)**, respectively.

not model-dependent since CAM-xenografted tumors displayed similar morphology to that of the mouse models in which the grafted cells were generated (**Figure 4B**) (Bierie et al., 2008; Forrester et al., 2005). Immunohistochemistry for phospho-Smad2 confirmed that TβRII$^{fl/fl}$ tumors maintained TGF-β signaling in epithelial and stromal cells, while TβRII KO tumors lacked signaling in epithelia only (**Figure 4C**). Furthermore, tumor-associated immune infiltrates were identified and evaluated histologically, revealing an increased monocytic population in TβRII KO tumors as compared to TβRII$^{fl/fl}$ tumors (**Figure 4D**). At the cellular level, it is apparent that strand migration and numerous single epithelial cells were visible at the tumor-stromal interface and tumor edges of TβRII$^{fl/fl}$ tumors (**Figures 4B, 5A**). In contrast, tumor cells at the tumor-stromal interface and tumor edges of TβRII KO tumors were visible as large clusters or cohorts. These findings corresponded with our observations during time-lapse imaging of cell migration (**Figure 1B**). One potentially confounding variable in our *in ovo* observations is the reproducibility with multiple xenografted cell lines. Using several carcinoma and fibroblast cell lines with the appropriate TβRII status, we therefore confirmed an identical pattern of single cell/strand migration (TβRII$^{fl/fl}$ tumors) or collective migration (TβRII KO tumors) (**Figure 5B**).

Numerous publications have demonstrated that differential modes of cell migration can correlate with altered metastatic ability. In order to distinguish differential metastasis of TβRII$^{fl/fl}$ or TβRII KO tumor cells, CAM distant from the primary tumor site was harvested from *in ovo* tumor-bearing animals. The amount of metastasis was then analyzed using murine-specific *Alu* PCR. Metastasis of collective aggregates in TβRII KO tumors was nearly 2.5-fold higher than that of TβRII$^{fl/fl}$ tumors (**Figure 6A**). In this way, collective migration of cells lacking TGF-β signaling appeared to present a distinct advantage over single cell/strand migration of cells in stromal invasion. In order to further substantiate our metastatic findings, an *in ovo* experimental metastasis assay was performed. Using murine-specific *Alu* PCR, this assay detects

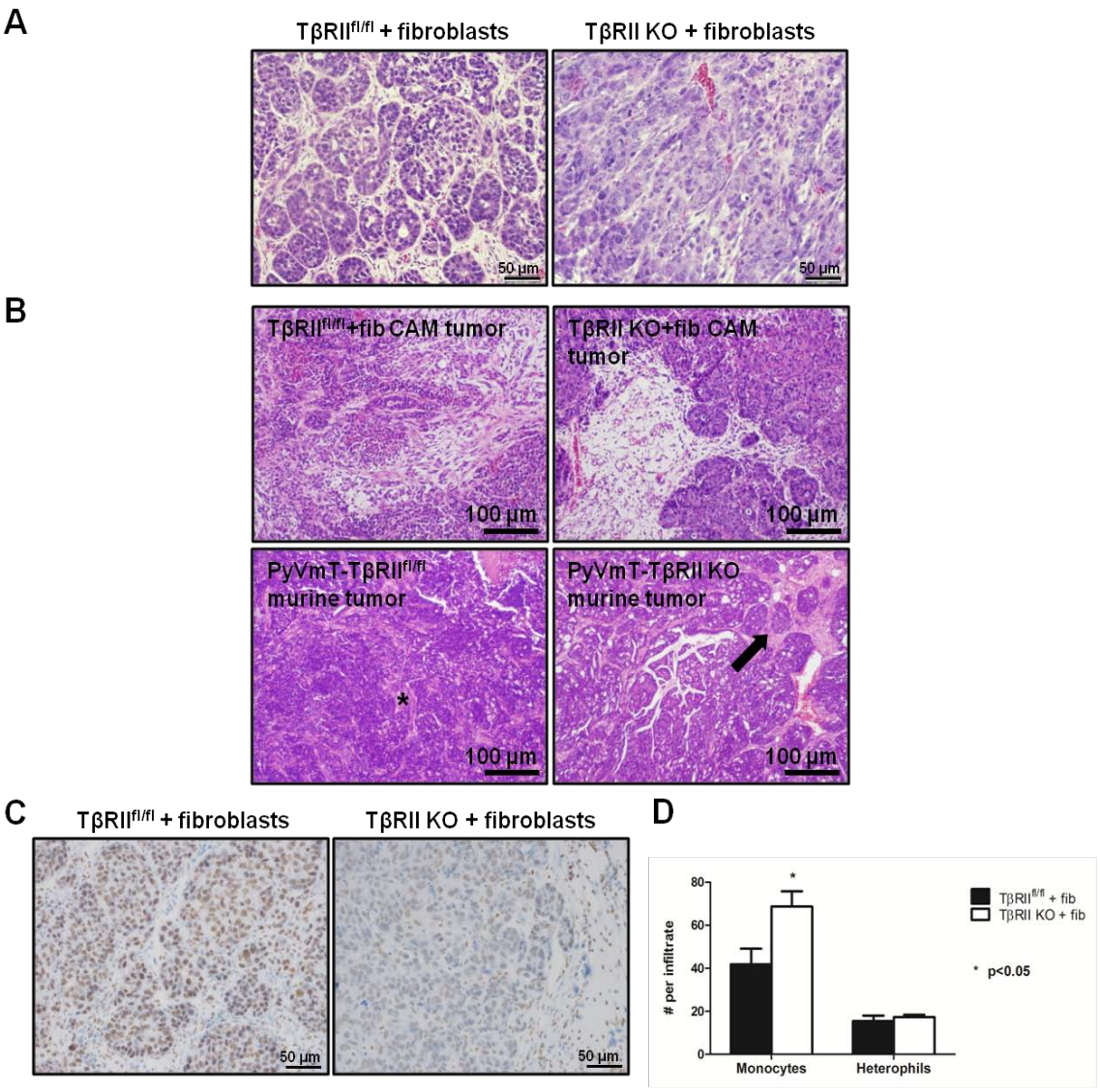

Figure 4. TβRII$^{fl/fl}$ and TβRII KO *in ovo* tumors were compared histologically.

(A) H&E sections of *in ovo* tumors revealed overall tumor histology. **(B)** H&E comparison of *in ovo* tumors compared to the corresponding mouse models from which the cells were derived. The tumor-stromal interface is shown. The asterisk (*) denotes an area of single cells in an invasive region, while the arrow indicates clustered cells in an invasive region. **(C)** TβRII$^{fl/fl}$ tumors maintain epithelial and stromal TGF-β signaling as indicated through phospho-Smad2 expression, while TβRII KO tumors maintain TGF-β signaling only in the partial TβRII KO fibroblasts. **(D)** Tumor-associated immune infiltrates of monocytes and heterophils were identified and evaluated in H&E sections. Infiltrates in TβRII KO tumors were characterized by increased monocytic populations as compared to TβRII$^{fl/fl}$ tumors.

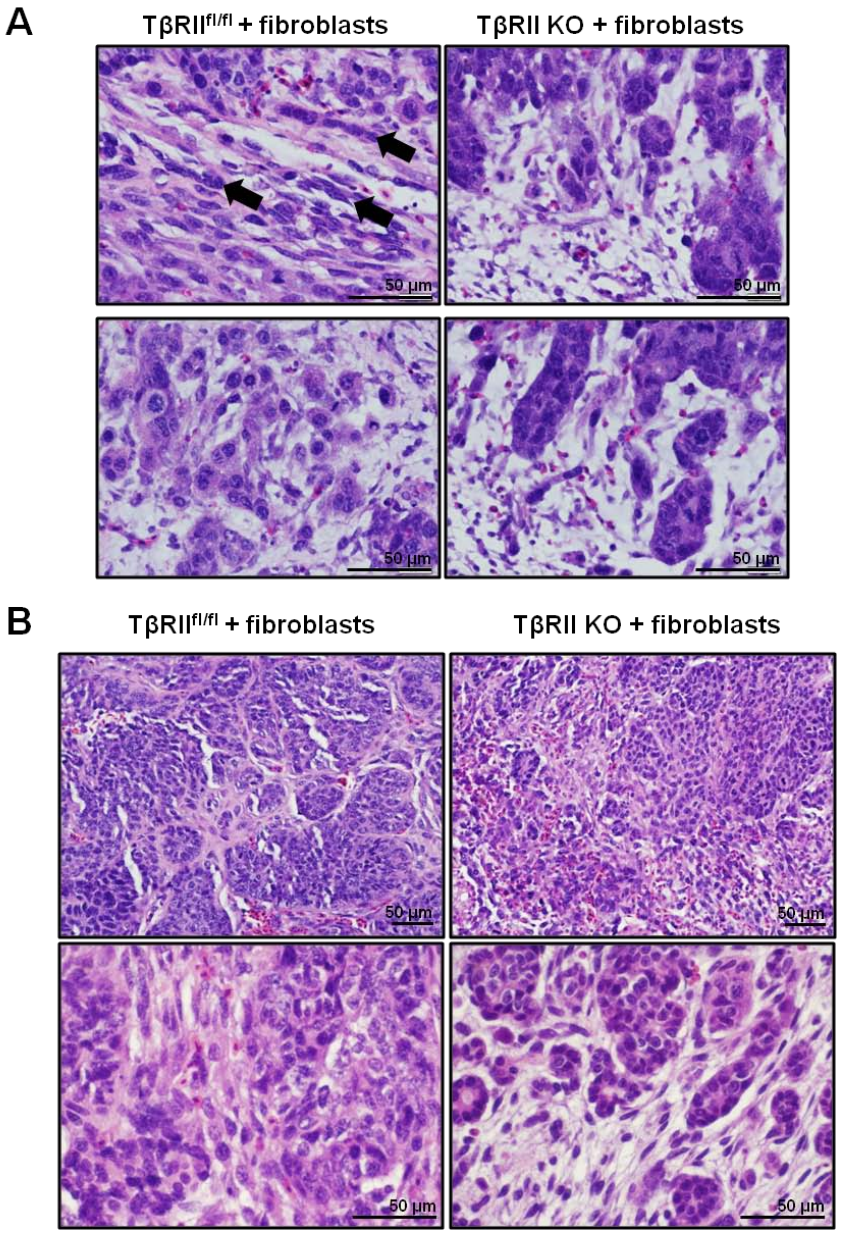

Figure 5. Single cell and collective cell invasive aggregates were observed in TβRII$^{fl/fl}$ and TβRII KO *in ovo* tumors.

(A) Evidence of strand filing (top left panel, arrows) and single cells (bottom left panel) were seen in H&E sections of TβRII$^{fl/fl}$ tumors. Collective clusters were seen in TβRII KO tumors. Pictures are representative of images at the tumor periphery and at tumor-stromal boundaries. **(B)** Additional TβRII$^{fl/fl}$ and TβRII KO epithelial cell lines were combined with fibroblasts to confirm similar *in ovo* histology as that observed in tumors detailed in this chapter. Overall histology (top panels) and single cell (bottom left panel) or collective migration (bottom right panel) are shown.

the presence of epithelial cells in the CAM initially upon vascular arrest and subsequently for extravasation and proliferative capability. TβRII$^{fl/fl}$ carcinoma cells combined with fibroblasts maintained similar cell quantities upon vascular arrest and 18 hours post-vasculature entry; however, the presence of these cells continued to decline over the course of the assay (**Figure 6B**). This decline was attributed to the inability of all cancer cells to survive in circulation and to the fact that fibroblast survival in circulation has not been well documented. In contrast to the behavior of the TβRII$^{fl/fl}$ cells and fibroblasts, although TβRII KO carcinoma cells combined with fibroblasts resulted in a similar initial cell decline, there was a subsequent increase for the duration of the assay. This steady rise was attributed to better extravasation, survival, and colonization abilities of TβRII KO epithelia. This finding corroborates the CAM metastasis results suggesting that the collective TβRII KO aggregates are better capable of metastasis (**Figure 6A**). In both cell combinations, it was also observed that the majority of extravasated cells were present in clusters near vasculature, with the TβRII KO epithelia forming more compact clusters (**Figure 6B**). The vascular proximity of colonizing cells supports our *in ovo* migratory results demonstrating directional vasculature migration (**Figure 2C**). As confirmation of our extravasation results, an additional experimental metastasis assay was completed using carcinoma cells alone (**Figure 7A**). Although the presence of TβRII$^{fl/fl}$ epithelial cells remained constant over the course of the assay, the TβRII KO epithelia were better able to extravasate and survive; however, neither the TβRII$^{fl/fl}$ nor the TβRII KO epithelia had evidence of invasive cellular protrusions present when epithelial cells were combined with fibroblasts (**Figures 6B, 7A**). When combining these two separate experimental metastasis assays, it suggests that the carcinoma cells may innately possess an extravasation ability that is enhanced by fibroblast presence. Investigation of intravasation capability, the initial step in metastatic dissemination, revealed no differences between the TβRII$^{fl/fl}$ and TβRII KO epithelial cells (**Figure 7B**).

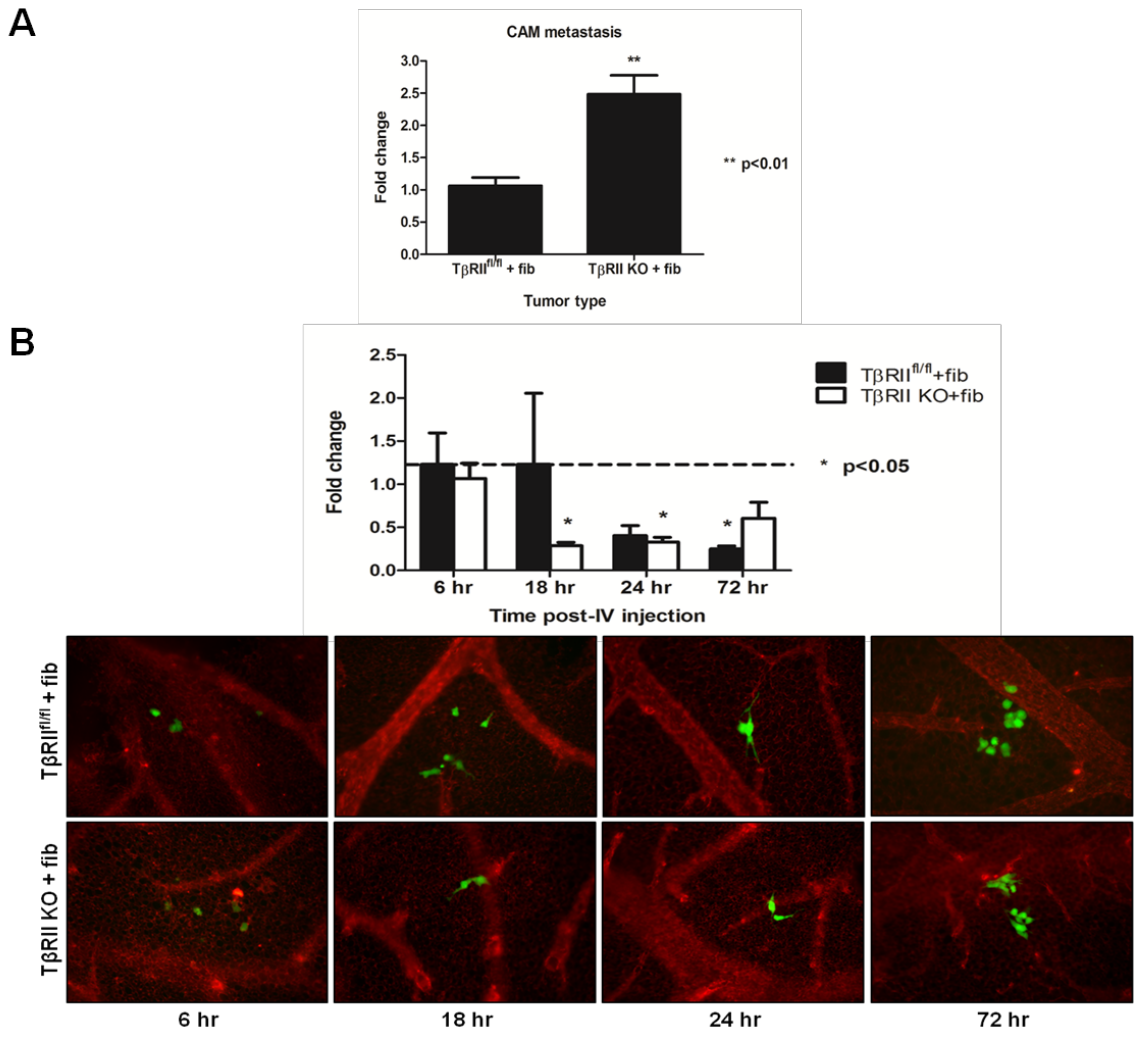

Figure 6. Single cell and collective cell invasive aggregates demonstrated different extravasation and metastatic potentials.

(A) Results from murine-specific *Alu* qPCR found that collective aggregates of TβRII KO tumors achieved greater metastasis than single cells of TβRII$^{fl/fl}$ tumors *in ovo*. **(B)** TβRII KO epithelial cells possess a greater ability than do TβRII$^{fl/fl}$ cells to extravasate and survive post-extravasation. This was quantified via an experimental metastasis assay and subsequent murine-specific *Alu* PCR (top graph). All time points and samples were compared to the 6 hour time point of the TβRII$^{fl/fl}$ cells and fibroblasts (*dashed line*). Representative images of epithelial cells (green) in relation to the lectin-labeled vasculature (red) were taken at all time points to confirm extravasation quantification and are shown beneath the graph (fibroblasts were unlabeled and therefore not shown). The 6 hour time point represented arrested cells in the vasculature. Presence of carcinoma cells in the capillary bed, which is porous, was seen. At the 18 and 24 hour time points, proliferative capability of disseminated tumor cells was seen. This was evident in cells extravasating from the capillary bed, invading into areas of the CAM in close proximity to the vasculature, and exhibiting protrusive cellular processes. At the 72 hour time point, cohesive groups of cells with protrusive cellular processes were observed near vessels.

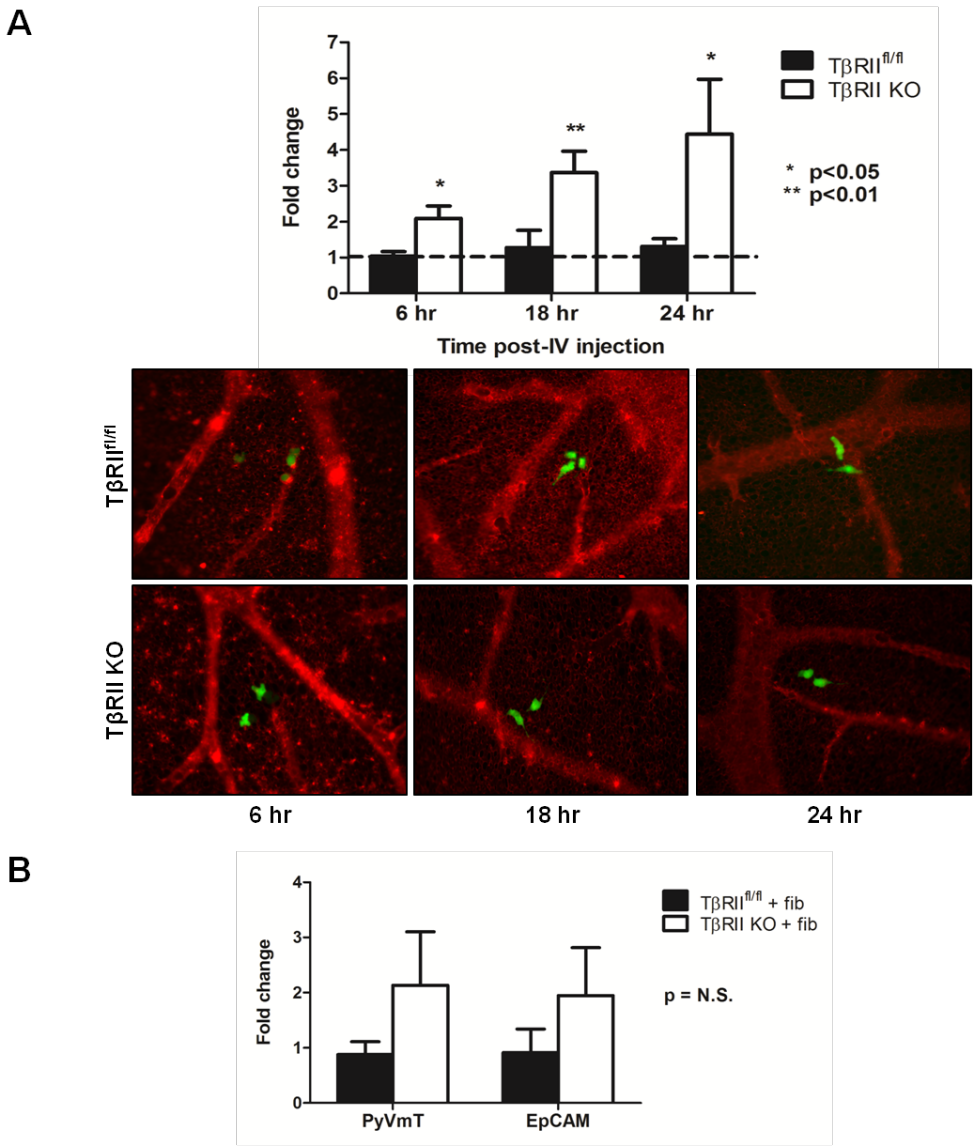

Figure 7. TβRII KO epithelial cells possessed a greater ability than did TβRII$^{fl/fl}$ cells to extravasate and survive post-extravasation.

(A) Extravasation quantification was completed via an experimental metastasis assay and subsequent murine-specific *Alu* PCR (top graph). All time points and samples were compared to the 6 hour time point of the TβRII$^{fl/fl}$ cells (*dashed line*). Representative images of epithelial cells (green) in relation to the lectin-labeled vasculature (red) were taken at all time points to confirm extravasation quantification and are shown beneath the graph. The 6 hour time point represented arrested cells in the vasculature. Presence of carcinoma cells in the capillary bed, which is porous, was seen. At the 18 and 24 hour time points, proliferative capability of disseminated tumor cells was seen. This was evident in cells extravasating from the capillary bed and invading into areas of the CAM in close proximity to the vasculature. **(B)** Intravasation of epithelial cells from TβRII$^{fl/fl}$ and TβRII KO tumor-bearing chicken embryos was quantified. Analysis compared expression of PyVmT and EpCAM, both epithelial markers.

In order to confirm that the observed migratory phenotypes were TβRII-dependent, TβRII KO epithelial cells were reconstituted with functional TβRII (RII) to regain responsiveness to TGF-β signaling (**Figure 8A**) (Xi et al., 2008). *In ovo* xenografts of $T\beta RII^{fl/fl}$, TβRII KO, or TβRII KO+RII were combined with fibroblasts, and migratory phenotype of the tumor cells was observed. Indeed, TβRII KO+RII epithelia showed evidence of single cell migration at the tumor periphery, thereby recapitulating the migratory phenotype observed in $T\beta RII^{fl/fl}$ tumors (**Figure 8B**). These results substantiated the conclusion that single cell versus collective cell migration was a consequence of TβRII expression.

Epithelia lacking TGF-β signaling maintain junctional protein localization at the tumor-stromal interface

During development and tumorigenesis, it is sometimes necessary for cells to maintain polarity and junctional adherence, albeit somewhat transiently (Ewald et al., 2008; Friedl and Gilmour, 2009). This is important to effective forward migration of epithelial sheets during organ formation, as well as increased pressure of tumor epithelia to push against surrounding stroma during tumor proliferation. The divergent individual versus collective migratory phenotypes of $T\beta RII^{fl/fl}$ and TβRII KO tumor cells observed in real-time imaging and in histological sections suggest that molecular distinctions responsible for cell-cell adhesion and migration are developed in response to TGF-β signaling. Indeed, immunohistochemical results indicated that E-cadherin expression was highly mislocalized in epithelia at the tumor-stromal interface of $T\beta RII^{fl/fl}$ tumors (**Figure 9A**). Higher magnification revealed maintenance of E-cadherin membrane localization in multicellular lobular tumor structures but cytoplasmic localization or potential degradation in single epithelial cells. This contrasted with E-cadherin membrane

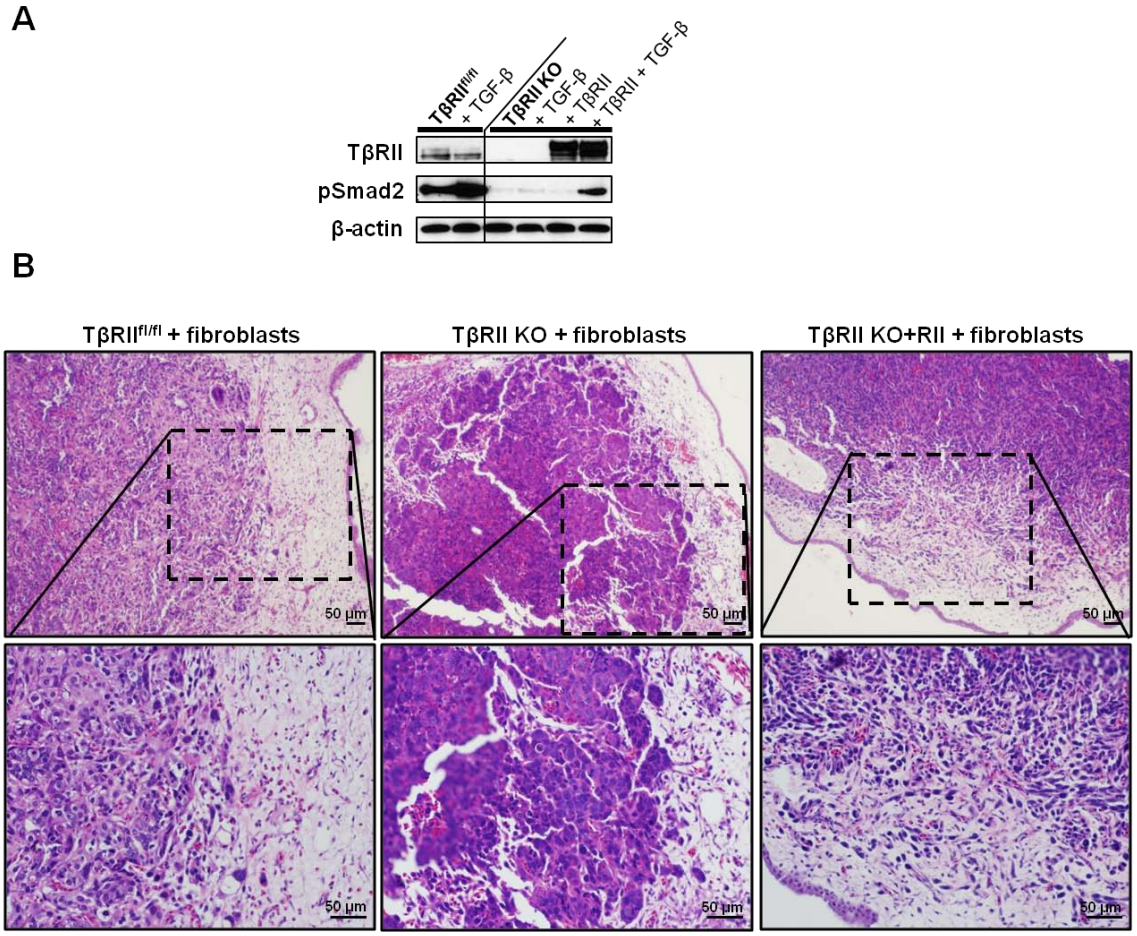

Figure 8. Single cell migration was a TβRII-dependent event.

(A) TβRII KO cells used for xenografting were reconstituted with functional hTβRII. TβRII$^{fl/fl}$ cells were used as a control for active TGF-β signaling as assessed by phospho-Smad2 expression. A shorter exposure of the hTβRII blot was used for all TβRII KO lanes due to overexpression signal strength. **(B)** Reconstitution of active TGF-β signaling in TβRII KO epithelia recapitulated the single cell migratory phenotype observed in TβRII$^{fl/fl}$ tumors.

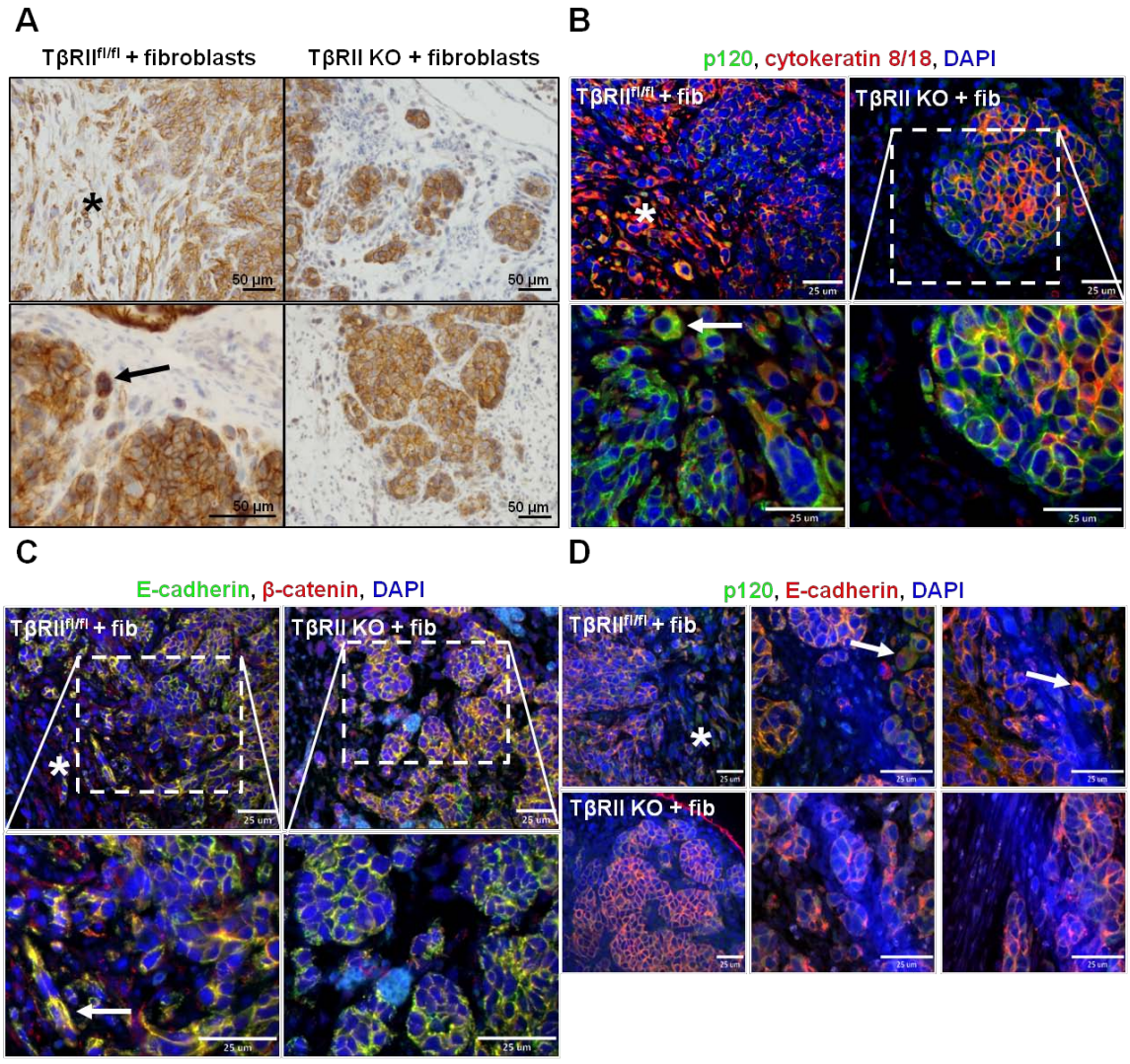

Figure 9. TGF-β signaling in epithelial cells disrupted maintenance of E-cadherin/p120/β-catenin membrane localization at adherens junctions.

All images were taken of *in ovo* tumors (*asterisks* designate tumor-stromal regions; *arrows* denote single cells with protein mislocalization). **(A)** Immunohistochemistry showed that E-cadherin was mislocalized in tumor-stromal regions in which single cells were found in TβRII$^{fl/fl}$ tumors (top and bottom left panels). Collective clusters in the same regions exhibited E-cadherin membrane localization in TβRII KO tumors (top and bottom right panels). **(B-D)** Immunofluorescence for p120, E-cadherin, and β-catenin revealed mislocalization of their expression in stromal areas of TβRII$^{fl/fl}$ tumors but maintenance in TβRII KO tumors. Cytokeratin 8/18 was used as a marker for carcinoma cell identification.

localization in all collective clusters at the tumor-stromal interface of TβRII KO tumors. In order to further analyze junctional characteristics of the tumor types, cytokeratin 8/18 was used in immunofluorescence to distinguish epithelial cells from surrounding stromal cells. Results indicated that p120 and β-catenin were mislocalized in TβRII$^{fl/fl}$ epithelia that possess TGF-β signaling, corresponding to the mislocalized E-cadherin evident in these tumors (**Figures 9B, 9C, 9D**). On the other hand, E-cadherin expression in clusters of TβRII KO tumors co-localized with both p120 and β-catenin expression at the membrane, suggesting maintenance of adherens junctions. Similarly, tight junctions also remained intact in TβRII KO tumors, as assessed by ZO-1 membrane localization, but were not maintained in TβRII$^{fl/fl}$ tumors at the tumor-stromal interface (**Figure 10A**).

Since epithelial clusters in TβRII KO tumors maintained junctional protein expression, and epithelia of TβRII$^{fl/fl}$ tumors appeared more mesenchymal, EMT-like markers were explored. As expected, epithelia in TβRII$^{fl/fl}$ tumors, marked by cytokeratin 8/18, expressed α-smooth muscle actin (α-SMA) and vimentin at the tumor-stromal interface and at the edges of lobular tumor structures (**Figures 10B, 10C, 10D**), confirming a mesenchymal phenotype. These observations are consistent with the idea that single cell migration may rely on classical mechanisms of EMT, such as loss of adherens and tight junctions and reorganization of actin stress fibers, to drive tumor cell invasion. Interestingly, all collective clusters in TβRII KO tumors were immediately surrounded by vimentin-positive adjacent fibroblasts. This finding corroborates our *ex ovo* findings (**Figure 1B**) and previous studies suggesting fibroblast-led migration of epithelial cells (Gaggioli et al., 2007).

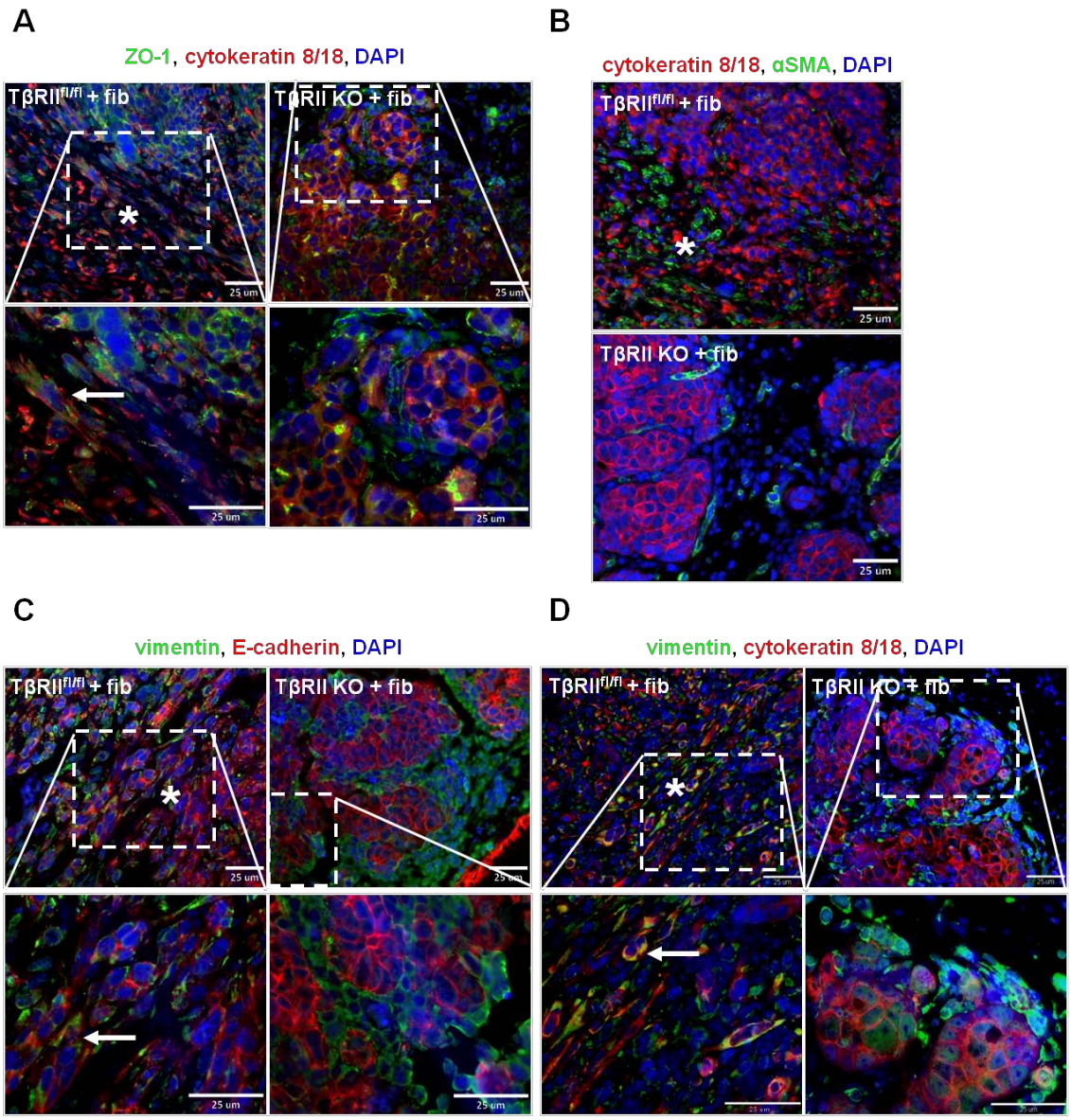

Figure 10. TGF-β signaling in epithelial cells disrupted tight junction protein localization while enhancing migratory protein expression.

All immunofluorescent images were taken of *in ovo* tumors (*asterisks* designate tumor-stromal regions; *arrows* denote single cells with protein mislocalization). Cytokeratin 8/18 was used as a marker for carcinoma cell identification. **(A)** ZO-1 was mislocalized in stromal areas of TβRII$^{fl/fl}$ tumors but maintained in TβRII KO tumors. **(B-D)** Increased expression of α-SMA and vimentin was seen in TβRII$^{fl/fl}$ tumor cells located in tumor-stromal areas. Vimentin was expressed by fibroblasts immediately surrounding TβRII KO epithelial clusters.

Differing migration modes are associated with gene expression differences in *in ovo* tumors

In order to identify gene expression changes that contribute to motility and invasion in response to loss of TGF-β signaling, we isolated tumor cells at the tumor-stromal interface using laser capture microdissection (LCM) on frozen *in ovo* tumor sections. For TβRII$^{fl/fl}$ tumors, single migratory epithelial cells and epithelia lining the tumor-stromal interface were captured (**Figures 11A, 11B**). For TβRII KO tumors, migratory epithelial clusters in the stroma and epithelia lining the tumor-stromal interface were captured. Samples were then analyzed on an EMT qPCR array (**Figure 12A**). Epithelial purity of the LCM samples was confirmed via PyVmT and EpCAM expression in comparison to FAP expression, markers of epithelia and fibroblasts, respectively (**Figure 12B**). It is important to note that the epithelial markers were similarly expressed in both TβRII$^{fl/fl}$ and TβRII KO LCM samples, indicating the same quantity of epithelia in all LCM samples (**Figure 12C**). Using a ten-fold or greater upregulation or downregulation stringency for the EMT array, we identified upregulation of Cdh2, Igfbp4, and Tspan13, as well as downregulation of Col1α2, Bmp7, Wnt11, Gng11, Vcan, Tmeff1, and Dsc2 in TβRII KO epithelia compared to TβRII$^{fl/fl}$ epithelia (**Figure 12D**). These target genes shared integral roles in cell-cell binding and growth factor signaling. Target expression was validated via immunoblot for N-cadherin, Vcan, and Tmeff1 (**Figure 13A**). Additionally, target expression of Wnt11, Tmeff1, and Dsc2 was confirmed via qPCR on the cultured cell lines used for the *in vivo* assays (**Figure 13B**). Interestingly, the presence of fibroblast conditioned media induced similar gene expression changes to those seen by the LCM epithelia that were in the physical presence of fibroblasts. We also investigated some genes frequently associated with collective (DDR1, eIF4GI) (Hidalgo-Carcedo et al., 2011; Silvera et al., 2009) and mesenchymal migration (Snai3) but found no significant expression difference between our tumor types (**Figure 13C**).

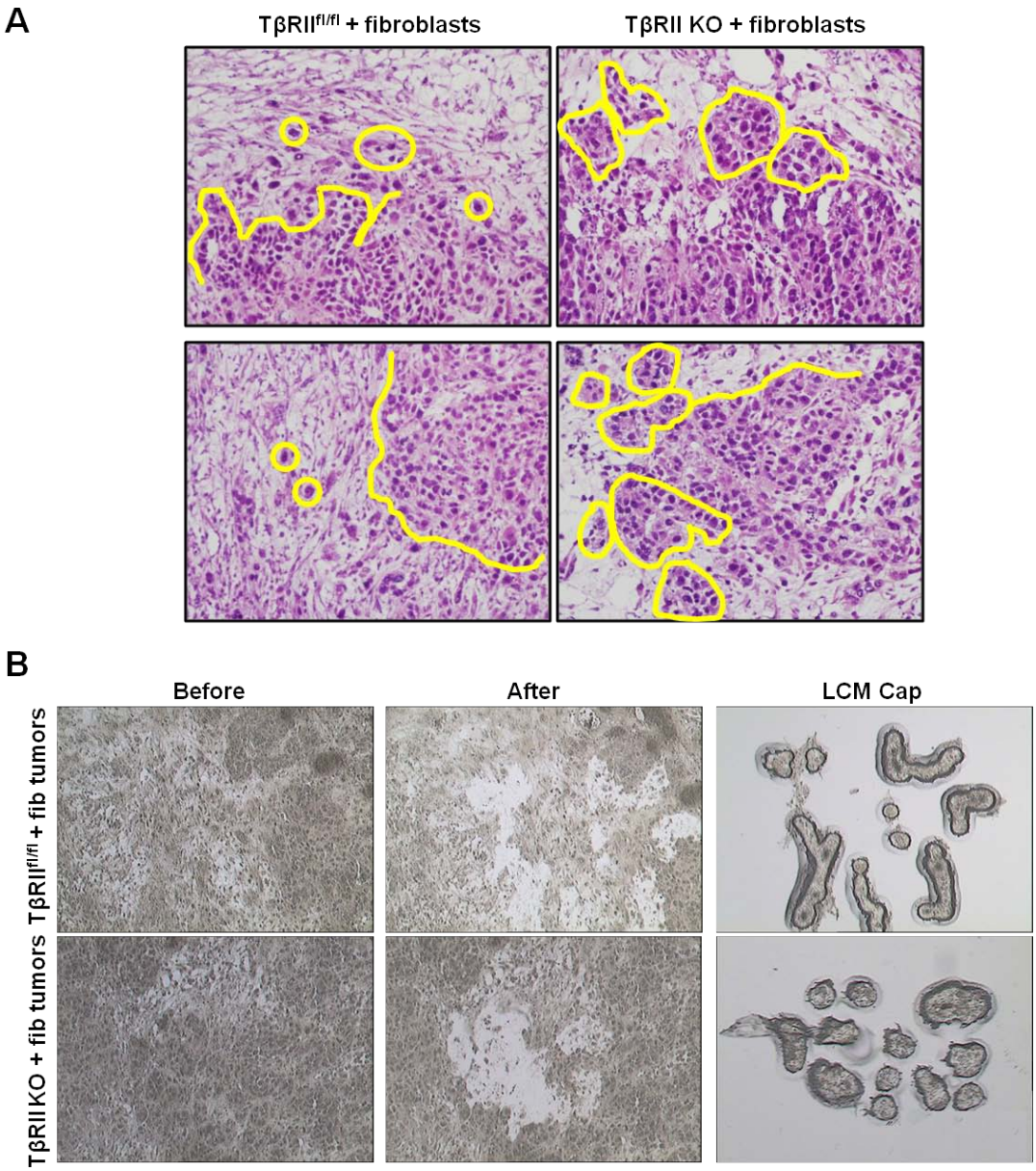

Figure 11. Laser capture microdissection (LCM) of *in ovo* tumor epithelia is demonstrated.

(A) Representative H&E sections of *in ovo* tumors are shown. Circled and highlighted areas of the tumor indicate which carcinoma cells were chosen for LCM isolation. **(B)** Sections of *in ovo* tumors are shown prior to (left panels) and after (middle panels) laser capture microdissection. The material obtained on the LCM cap is also shown (right panels).

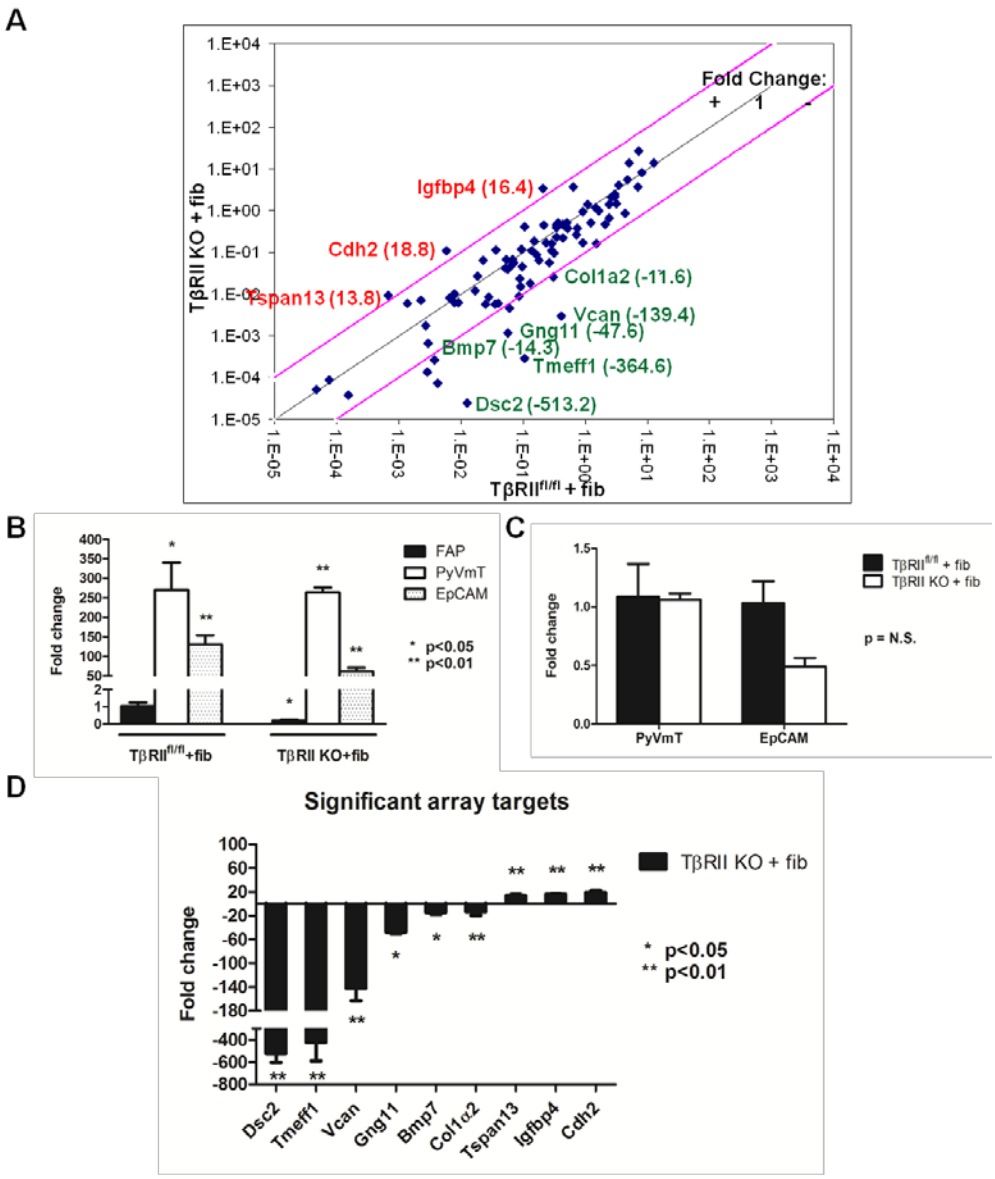

Figure 12. EMT gene expression changes were seen between tumors differing in invasive phenotype.

Gene expression changes detected on an EMT qPCR array were determined upon comparison of TβRII KO isolated epithelia to TβRII$^{fl/fl}$ isolated epithelia. **(A)** Identification of target genes was found by fold change values. All highlighted genes were statistically significant ($p<0.05$) and conform to the criteria of either 10-fold or greater up- or downregulation when comparing the TβRII KO LCM epithelia with the TβRII$^{fl/fl}$ epithelia. **(B)** Epithelial purity of all LCM samples was confirmed when comparing PyVmT or EpCAM epithelial marker expression to that of the FAP fibroblast marker. All expression values were compared to FAP expression in the TβRII$^{fl/fl}$ LCM sample. **(C)** Similar amounts of epithelia, as quantified by expression of PyVmT and EpCAM epithelial markers, were found in TβRII KO and TβRII$^{fl/fl}$ LCM samples. **(D)** Array target gene expression (identified in (A)) of TβRII KO LCM samples, as compared to that of TβRII$^{fl/fl}$ LCM samples, is shown with associated statistics.

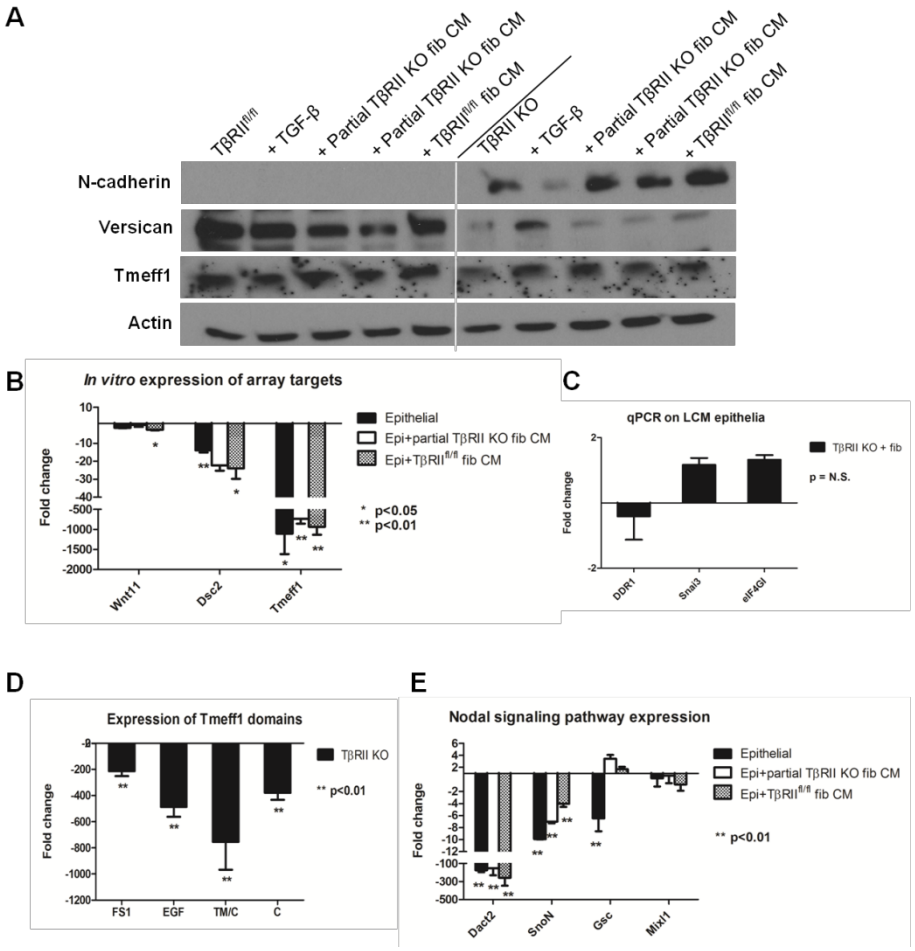

Figure 13. EMT gene expression changes were confirmed using cultured cells.

Cells used were the same as those xenografted onto the CAM (Figure 1). **(A)** Target gene validation was confirmed by immunoblotting. For either the TβRII$^{fl/fl}$ cells or the TβRII KO cells, the conditions were as follows: cells alone, cells treated with 1 ng/mL TGF-β for 2.5 hours, cells treated with partial TβRII KO fibroblast conditioned media for 24 hours (two cell lines used), or cells treated with TβRII$^{fl/fl}$ fibroblast conditioned media for 24 hours. Conditioned media treatment from partial TβRII KO and TβRII$^{fl/fl}$ fibroblasts gave similar results. **(B)** Wnt11, Tmeff1, and Dsc2 expression in TβRII KO cells paralleled results seen in array results. For each condition (epithelial cells alone or fibroblast conditioned media treatment), all TβRII KO cell samples were respectively compared to TβRII$^{fl/fl}$ cells. **(C)** No significant differences in DDR1, Snai3, or eIF4GI expression between TβRII$^{fl/fl}$ and TβRII KO LCM tumor epithelia were seen via qPCR analysis. Only expression fold changes of TβRII KO LCM epithelia, as compared to TβRII$^{fl/fl}$ LCM epithelia, are shown. **(D)** Expression levels of Tmeff1 domains were analyzed. Only expression fold changes of TβRII KO cells, as compared to TβRII$^{fl/fl}$ cells, are shown. FS1 – follistatin 1, EGF – epidermal growth factor, TM/C – transmembrane and cytoplasmic, C – cytoplasmic. **(E)** Expression of Nodal signaling inhibitors (Dact2, SnoN) was downregulated but unaccompanied by significant expression increases of Nodal targets, with the exception of Gsc (comparing epithelial cells alone). For each condition (epithelial cells alone or fibroblast conditioned media treatment), all TβRII KO cell samples were respectively compared to TβRII$^{fl/fl}$ cells.

One of the targets, Tmeff1, is a type I transmembrane receptor with signal transduction activity and is known to play a role in cancer progression signaling through induction of erbB4 tyrosine kinase receptor phosphorylation (Uchida et al., 1999) and suppression of Nodal signaling. Tmeff1 inhibits Nodal signaling via binding to the Nodal co-receptor, Cripto (Harms and Chang, 2003), which is overexpressed in ~70-80% of invasive human breast cancer (Panico et al., 1996; Qi et al., 1994). It has previously been shown that increased expression of Tmeff1 is a direct result of Smad-dependent TGF-β signaling in the hair follicle (Oshimori and Fuchs, 2012). Analysis of Tmeff1 domains (Chang, 2012; Chang et al., 2003) in cultured TβRII KO epithelial cells compared to cultured $T\beta RII^{fl/fl}$ epithelial cells revealed significant downregulation of all domains (**Figure 13D**). Given that Tmeff1 is just one of several Nodal pathway inhibitors, we explored the expression of these other inhibitors. Dact2, which binds to activin type I receptors and targets them for lysosomal degradation, was ≥50-fold downregulated in TβRII KO epithelia across all *in vitro* conditions tested (**Figure 13E**). Downregulation of SnoN, an inhibitor of Nodal and TGF-β signaling, was also seen. Due to the observed downregulation of Nodal inhibitors, it might be inferred that activation of Nodal target genes would result. Surprisingly, only the Nodal target Gsc was upregulated in TβRII KO epithelia, while several other target genes (Nodal, Lefty 1/2, Ubr7, HESX1, Moap1, Cer1) were unaffected (**Figure 13D**, data not shown).

Discussion

Patterns of carcinoma cell migration strikingly resemble those in development, organogenesis, tissue remodeling, and wound healing. During early embryogenesis, EMT is frequently observed in gastrulation, while in late embryogenesis, EMT is characteristic of neural crest migration (Micalizzi et al., 2010; Moustakas and Heldin, 2007). Collective migration of

epithelial sheets generates solidified epithelial barriers in organ development. Some of these sheets are led by tip cells that serve as a communication conduit to following cells in the cohort (Revenu and Gilmour, 2009). In mammary branching morphogenesis, the development and elongation of the mammary ductal tree involves collective invasion of terminal end buds (Ewald et al., 2008; Friedl and Gilmour, 2009). Epithelial sheets and clusters maintain apicobasal polarity and cell-cell junctions. In these examples of cellular processes, cooperation is required between multiple cell populations, such as epithelial-stromal crosstalk. Evidence of both EMT and cohesive invasion can be found in our model of epithelial-stromal interactions within the tumor microenvironment. Fibroblasts were required for carcinoma cell invasion, suggesting a microenvironmental component of cellular communication. Our cohesively moving TβRII KO epithelia maintained adherens and tight junctional proteins necessary for cell-cell adhesion. Tip cells within these clusters have expressed vimentin, providing credence to transient mesenchymal characteristics within the forefront of collectively invading epithelia (Friedl and Gilmour, 2009). Similar to EMT phenotypes seen in development, our $T\beta RII^{fl/fl}$ tumors with competent TGF-β signaling express α-SMA and vimentin and lose junctional polarity.

The predominant perception of TGF-β signaling in tumor migration has been that TGF-β induces single cell invasion that is correlated with increased invasive and metastatic potential. This has commonly been associated with epithelial cells undergoing EMT by which they acquire mesenchymal characteristics of stromal cells and presumably become invasive. Yet, recent evidence from *in vitro* studies finds a collective migration component of tumors (Gaggioli et al., 2007). There is histological evidence of chain or collective epithelial cell migration in human cancer. For many years, pathologists have identified cohorts of cells in stromal areas surrounding primary tumors (Christiansen and Rajasekaran, 2006). In many instances, epithelial movement occurs within the epithelial-stromal interface of the tumor itself or at the tumor

periphery. Consistent with current views, our work suggests that the presence of epithelial TGF-β signaling causes a single cell or strand migration. On the other hand, a lack of epithelial TGF-β signaling induces a collective tumor invasive front in the tumor areas prone to increased cell movement. Fibroblasts were able to induce these two varying patterns of migration. This suggests a pro-migratory effect provided by stromal fibroblasts that enables a cell-autonomous epithelial response dependent upon TGF-β signaling capability. A lack of TGF-β signaling has previously been implicated in collective migration, but this was shown through exogenous manipulation of the TGF-β pathway (Giampieri et al., 2009a). Our results, using genetic, cell-autonomous control of TGF-β signaling through expression of TβRII, specifically identified TGF-β as a critical factor involved in epithelial migration in the tumor microenvironment. The novelty of our findings also extended to the methodology by which we have achieved these results. Conventional *in vivo* imaging techniques afford minimal imaging length and significant viability issues inflicted on the animals used. The use of our cells in the CAM model enabled prolonged imaging and minimal embryo damage at each timepoint used for video capture.

A fluidity and plasticity between migration patterns is crucial to cancer progression. Beyond the characterization of tumor behavior at the primary site, the concept of mesenchymal-to-epithelial transition (MET) at secondary tumor sites has emerged (Hugo et al., 2007; Lee et al., 2006; Thiery, 2002). In MET, colonized metastases are histopathologically similar to the epithelial nature of the primary tumors from which they are derived (Brabletz et al., 2001; Tsuji et al., 2009). These metastases possess polarity markers and a re-epithelialization that maintains junctional protein expression. This is evident in the movement of metastatic emboli, or clustered epithelia, which are a hallmark of inflammatory breast cancer (Silvera et al., 2009). Our work supports the epithelial nature of invasive cell movement. The collective aggregates observed in TβRII tumors were capable of greater CAM metastasis than

were cells migrating singly or in strands that maintain TGF-β signaling. Additionally, our experimental metastasis assay results demonstrate that cells lacking TGF-β signaling possess an enhanced ability to extravasate, survive, and re-epithelialize at metastatic sites. The ability to colonize at distant sites, regardless of TβRII expression and cell quantity, is supporting evidence for MET. Since no difference in intravasation ability was found between tumors with and without TGF-β signaling, our results suggest that the extravasation and survival steps of the metastatic cascade may be where cells lacking TGF-β signaling have a distinct advantage in positively contributing to metastasis.

Our results begin to pinpoint a mechanism responsible for the clustered TβRII KO epithelial invasion versus the single cell or strand migration of TGF-β-competent epithelia. Tmeff1 is a crucial inhibitor of the Nodal signaling pathway, which is responsible for many EMT-related effects. It is therefore noteworthy that our TβRII KO epithelia significantly downregulated Tmeff1 yet maintained a clustered aggregate formation during invasion. We showed that other Nodal signaling pathway inhibitors were also downregulated. Our results allude to a significant overlap between TGF-β and Nodal signaling pathways as a consequence of TβRII loss. Given that Tmeff1 contains Smad-binding elements in its promoter and has been shown to be activated in Smad-dependent TGF-β signaling in the hair follicle (Oshimori and Fuchs, 2012), it is likely that it is also a TGF-β target in the mammary gland, a question further being pursued. Tmeff1 may also be regulated by a fibroblast-secreted factor in the tumor microenvironment. Our results using fibroblast conditioned media suggest that the physical presence of fibroblasts may not be necessary to induce gene expression changes responsible for migration patterning. This corroborates previously published studies implicating the role of fibroblast-secreted factors in tumor cell proliferation and motility (Cheng et al., 2005; Xu et al., 2010).

Our findings illustrate a critical role for TGF-β signaling in the regulation of tumor microenvironmental interactions. Epithelial-stromal signaling deserves further study as a prominent driver of invasive and metastatic progression. The presence of fibroblasts induces specific carcinoma cell migration patterning dependent upon TGF-β competency. Further characterization of single cell versus collective cell migration is needed in tumor analysis in order to better understand the contribution of each to tumor progression. Upon further investigation, it is the hope that specific patterns of tumor invasiveness can be targeted as recourse for breast cancer treatment.

CHAPTER III

MOUSE MODEL OF TGF-β SIGNALING IN MAMMARY TUMORIGENESIS AND PROGRESSION

Introduction

The expansive effects of transforming growth factor-beta (TGF-β) on development, cellular homeostasis, and cancer underscore the global importance of this pleiotropic cytokine in multiple cell types (Bierie and Moses, 2006b; Massague, 2008; Taylor et al., 2011). This vast influence of TGF-β signaling thus renders the pathway a frequent target for dysregulation during cancer progression, particularly in breast cancers (Gobbi et al., 2000; Hinshelwood et al., 2007; Levy and Hill, 2006). Much of the current scientific understanding of this dysregulation has been achieved through elegant murine studies. In these studies, carcinoma cell autonomous TGF-β signaling has been explored through pathway activation, via ligand overexpression or constitutively active receptor expression, or pathway inactivation, via mutation or deletion of TGF-β ligand or receptors.

Studies investigating ablation of TGF-β pathway members, including all three TGF-β ligands and canonical signaling receptors, resulted in embryonic or perinatal mortality (Pangas and Matzuk, 2004). One of the first murine TGF-β models exploited ablation of *Tgfb1* (Dickson et al., 1995; Kulkarni et al., 1993; Shull et al., 1992). Mice with global loss of TGF-β1 ligand either died of yolk sac defects, attributed to defective hematopoiesis and vasculogenesis, or succumbed to a lethal wasting syndrome induced by inflammation and tissue necrosis. Lethality was also seen in TGF-β2-null mice exhibiting perinatal developmental and organ defects

(Sanford et al., 1997). Perinatal lethality was further evidenced in TGF-β3-null mice, which experienced delayed pulmonary development and defective palatogenesis (Kaartinen et al., 1995; Proetzel et al., 1995). Furthermore, studies ablating TβRI resulted in embryonic lethality due to defective vascular development, while ablation of TβRII induced hematopoietic- and vasculogenic-driven embryonic lethality (Larsson et al., 2001; Oshima et al., 1996). Since mice with homozygous deletions of *Tgfb1*, *Tgfb2*, *Tgfb3*, *Tgfbr1*, or *Tgfbr2* are all lethal, studies targeting the TGF-β pathway shifted to conditional transgene and null expression systems (Pangas and Matzuk, 2004).

Specifically in the mammary epithelium, murine systems exploiting conditional expression of TGF-β signaling mediators have provided extensive insight into carcinoma cell autonomous signaling. Both the mouse mammary tumor virus (MMTV) and whey acidic protein (WAP) promoters have been the two predominant gene promoters used to drive conditional gene expression. MMTV models drive expression in the mammary gland epithelium during development, pregnancy, lactation, and involution, while WAP-driven expression occurs from mid-pregnancy to early involution. MMTV models of TGF-β pathway alterations are frequently crossed with transgenic mice expressing oncogenic drivers for mammary tumor development, such as MMTV-TGF-α, MMTV-*c-Neu*, and MMTV-PyVmT (Guy et al., 1992a; Guy et al., 1992b; Matsui et al., 1990). MMTV-TGF-$\beta1^{S223/S225}$ mice, which have constitutively active TGF-β1 expression, exhibited a longer latency for tumor development when crossed with and compared to MMTV-TGF-α bigenic mice, while MMTV-TGF-$\beta1^{S223/S225}$; MMTV-*c-Neu* mice expressed higher levels of vimentin and activation of the MAPK/AKT pathway (Muraoka et al., 2003; Pierce et al., 1995). In contrast, an inducible MMTV-TGF-$\beta1^{S223/S225}$; MMTV-PyVmT model for constitutive TGF-β1 expression resulted in enhanced lung metastases (Muraoka-Cook et al., 2004). Collectively, these studies suggest a tumor suppressive role for TGF-β1 early in mammary tumor

development, while also suggesting TGF-β contribution to later stages of tumor progression. Although these reports explored the role of TGF-β ligand in mammary gland tumors, the precise timing of the TGF-β suppressor-promoter switch remained unknown.

Studies of carcinoma cell-derived TGF-β production and effects on tumor development and progression were furthered by investigations into cell autonomous attenuation or ablation of TGF-β signaling. Attenuation of TGF-β signaling using a dominant negative type II TGF-β receptor (dnTβRII) resulted in lobular alveolar hyperplasia and an increased rate of tumor formation in conjunction with a *TGF-α* transgene; however, decreased pulmonary metastasis resulted when dnTβRII was expressed along with a *c-Neu* transgene (Gorska et al., 2003; Siegel et al., 2003). These studies further substantiated the dichotomous role of TGF-β in tumorigenesis and progression. Mammary-specific ablation of the type II TGF-β receptor (TβRII) also supported the role of TGF-β as a tumor suppressor but challenged the dogma of TGF-β as a metastatic promoter. Conditional knockout of TβRII in mammary carcinoma cells expressing PyVmT, achieved via MMTV-Cre and WAP-Cre transgenes, led to decreased tumor latency; however, in contrast to attenuated TGF-β signaling models, TβRII ablation increased pulmonary metastasis (Bierie et al., 2008; Forrester et al., 2005). Differences in metastatic presentation stemming from either attenuation or ablation of TβRII lend credence to TGF-β signaling being both context- and cancer type-dependent.

Although carcinoma production of TGF-β and cell autonomous TGF-β signaling have been studied in the mammary gland, the timing of this signaling is still unknown. From a therapeutic standpoint, it is most advantageous to target TGF-β signaling when TGF-β serves as a promoter of mammary tumor progression and metastasis (Korpal and Kang, 2010). Pinpointing the timing of the suppressor-promoter switch therefore remains a critical link

between TGF-β-targeted therapy and the timing of its administration to patients. We therefore chose to investigate the temporal control of TGF-β signaling using an inducible dominant-negative TβRII (dnTβRII) transgenic mouse model. Our results indicate that attenuation of TGF-β signaling in mammary carcinoma cells prior to tumor formation can delay tumorigenesis but increase pulmonary metastasis. Furthermore, if TGF-β signaling attenuation occurs at or after the time of tumor formation, no effect on pulmonary metastasis is found. Increased lung metastasis in early TGF-β-attenuated tumors can be attributed to an influx of MDSCs as well as MCP-1/CCL2 secretion. Our data therefore demonstrates that inhibition of TGF-β signaling prior to tumorigenesis enhances malignant severity and metastatic spread of tumors.

Experimental Procedures

Mouse models

Dominant-negative TβRII mice were crossed with MMTV-PyVmT and MMTV-rtTA transgenic mice to produce dnTβRII^{+rtTA} experimental and dnTβRII^{-rtTA} control lines used for analysis (Frugier et al., 2005; Gunther et al., 2002; Guy et al., 1992a). All mice were treated with doxycycline hyclate (DOX) (2 mg/mL; Sigma-Aldrich) in 5% sucrose (Sigma-Aldrich) drinking water. The drinking solution was kept in amber water bottles for light protection and was changed every three days. Mice were housed and handled according to approved Institutional Animal Care and Use Committee protocols at Vanderbilt University Medical Center.

Lung whole mounts

Lungs were perfused with heparin in PBS, harvested, and fixed in 10% NBF overnight at room temperature on day 1 of this 4 day procedure. On day 2, lungs were dehydrated, put in xylene

for 1 hour, and changed into fresh xylene overnight. On day 3, lungs were rehydrated, washed in running tap water for 30 minutes, stained with Mayer's hematoxylin (Sigma-Aldrich), and then washed in running tap water for 5 minutes. The lungs were then destained in 1% v/v HCl made from a 12 N solution followed by washes in running tap water for 1 hour. Tissues were left in tap water overnight. On day 4, lungs were dehydrated, placed in xylene for 1 hour, and placed in Histo-Clear™ (National Diagnostics) until analyzed. Lung metastases were counted on a stereoscope (Olympus SZH10).

Histopathology

Tumors were harvested, fixed in 10% NBF, and then paraffin-embedded and sectioned by the Vanderbilt Translational Pathology Shared Resource.

Northern blot hybridization

Total RNA was isolated from frozen tumor samples using TRIzol (Invitrogen) and further purified using an RNeasy Mini Kit with RNase-Free DNase (both Qiagen). RNA was run on electrophoresis gel and subsequently transferred for blotting. The dnTβRII RNA was then detected by Northern blot hybridization as previously described (Gorska et al., 2003; Joseph et al., 1999). The cDNA probe for dnTβRII RNA was synthesized at a 55°C annealing temperature using Superscript III reverse transcriptase (Invitrogen) and the following primers, specifically targeted to include the c-Myc epitope tag: 5' CAAGTCGGTTAACAGTGATG 3' forward and 5' CAGATCTTCTTCAGAAATAAG 3' reverse. The cDNA probe was then ^{32}P-labeled for dnTβRII mRNA detection. Cylophilin was used as a loading control.

Immunoblotting

Protein lysate preparation from frozen mammary tumor samples and immunoblotting procedures were followed as previously described (Bierie et al., 2008). PVDF membranes were blocked in 5% milk in TBST and incubated with primary antibody overnight (phospho-Smad2, TβRII, and β-actin) or for 4 hours (GFP and c-Myc) at 4°C. The following primary antibodies were used: phospho-Smad2 (1:1000; Millipore AB3849), TβRII (1:4000; Santa Cruz Biotechnology, Inc. sc-400), GFP (1:5000; Abcam AB6556), c-Myc (1:5000; Abcam AB19234), and actin (1:4000; Sigma A 2066). Corresponding secondary HRP ImmunoPure® antibodies were used (1:5000; Pierce). Chemiluminescence detection of protein was completed using Western Lightning® ECL (PerkinElmer).

Flow cytometry

Mammary tumors were harvested and digested as previously described (Novitskiy et al., 2011). Briefly, tumors were digested in serum-free RPMI media with collagenase I (1 mg/mL; Sigma-Aldrich) and dispase® II (1 mg/mL; Roche) for 2 hours at 37°C. Tumor tissue was incubated with DNase I (2 U/µl; Calbiochem®), centrifuged, resuspended in sterile PBS, and lysed. Cells were labeled with fluorescently-conjugated antibodies (CD45 and CD326, BioLegend®; CD324 eBioscience). The cells were analyzed on an LSRII flow cytometer (Becton Dickinson), and the data was analyzed with FlowJo flow cytometry software. Lungs were perfused, harvested, and digested as previously described (Novitskiy et al., 2008). Briefly, lungs were digested in serum-free RPMI media with collagenase XI (0.7 mg/mL; Sigma-Aldrich) and type IV bovine pancreatic DNase (30 µg/mL; Sigma-Aldrich) for 45 minutes at 37°C. Processing and analysis of lung tissue was then performed as described above for mammary tumors.

Cytokine antibody array

After isolation from respective murine tumors, flow-sorted CD45-;EpCAM+ control (dnTβRII^{-rtTA}) epithelial cells and CD45-;GFP+;EpCAM+ experimental (dnTβRII^{+rtTA}) epithelial cells were used. These cells were sorted respectively from three control and three experimental tumors. Cells were cultured overnight and were additionally treated with DOX (0.5 μg/mL) during this time, allowing dnTβRII^{+rtTA} cells to maintain dnTβRII expression. Conditioned media from cultured cells was collected 22 hours post-sorting, incubated, and detected according to the RayBio® Mouse Cytokine Array C2000 protocol (RayBiotech, Inc.). Densitometry analysis was used to normalize samples to positive controls. Normalized signal intensity was then used to assess fold change in cytokine secretion between control and experimental murine conditioned media samples. After removal of the conditions media from the cultured cells, RNA from the cells was collected as described below.

Expression analysis

Total cell RNA from 22 hour post-sorted epithelial cells (see above) was collected using TRIzol (Invitrogen) and further purified using an RNeasy Mini Kit with RNase-Free DNase (both Qiagen). cDNA was synthesized using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) as described by the manufacturer. A Bio-Rad CFX96 machine was used for qPCR employing SsoAdvanced SYBR® Green Supermix (Bio-Rad). The following primer sequences were used to amplify murine coding sequences of interest: IL-6 (5' TCCTCTCTGCAAGAGACTTCCATCC 3' forward, 5' GGGAAGGCCGTGGTTGTCACC 3' reverse), MCP-1/CCL2 (5' CCTGTCATGCTTCTGGGCCTGC 3' forward, 5' TGGGGCGTTAACTGCATCTGGC 3' reverse), MIP-1γ/CCL9 (5' TGGGCCCAGATCACACATGCAAC 3' forward, 5' CAAGCCCTTGCTGTGCCTTCA 3' reverse), EGF (5' GTGCATCCAAGGGCCAGGAGAC 3' forward, 5' CAGCACCCGGTCGGAACCTTG 3' reverse), MMP-3

(5' AGGTGGACCTAGAAGGAGGCAGC 3' forward, 5' CAGCCACAGCAGGACCGGAA 3' reverse), and TWEAK R/Fn-14 (5' CTTCTGCCTGGGATGCGCCG 3' forward, 5' CCAGCGCCAAAACCAGGACCA 3' reverse). C_t values were subjected to statistical analyses after normalization to GAPDH.

Statistics

All statistical analyses were reported using two-tailed unpaired *t* tests to determine significance ($p<0.05$).

Results

Dominant-negative TβRII expression was detected in the mammary gland

Temporal control of TGF-β signaling in a transgenic mouse model was achieved by utilizing dominant-negative TβRII (dnTβRII) transgenic mice (Frugier et al., 2005). These mice carry a bidirectional tetracycline-transactivator responsive promoter controlling expression of both enhanced green fluorescent protein (EGFP) and cytoplasmically truncated TβRII, which is tagged by a human c-Myc epitope. When crossed with MMTV-rtTA mice in the presence of DOX, dnTβRII expression in the mammary gland results and can therefore be identified via EGFP or c-Myc expression (Gunther et al., 2002). Additionally, MMTV-PyVmT mice were crossed into this model to serve as the oncogenic stimulus (Guy et al., 1992a). Both control ($dnT\beta RII^{-rtTA}$) and experimental ($dnT\beta RII^{+rtTA}$) animals contained the MMTV-PyVmT and dnTβRII transgenes; however, the MMTV-rtTA transgene was absent in $dnT\beta RII^{-rtTA}$ animals, rendering these mice incapable of dnTβRII expression (**Figure 14A**).

In order to reduce potential variability in tumorigenicity between $dnT\beta RII^{-rtTA}$ and $dnT\beta RII^{+rtTA}$ animals as a result of DOX treatment, all animals were uniformly administered DOX.

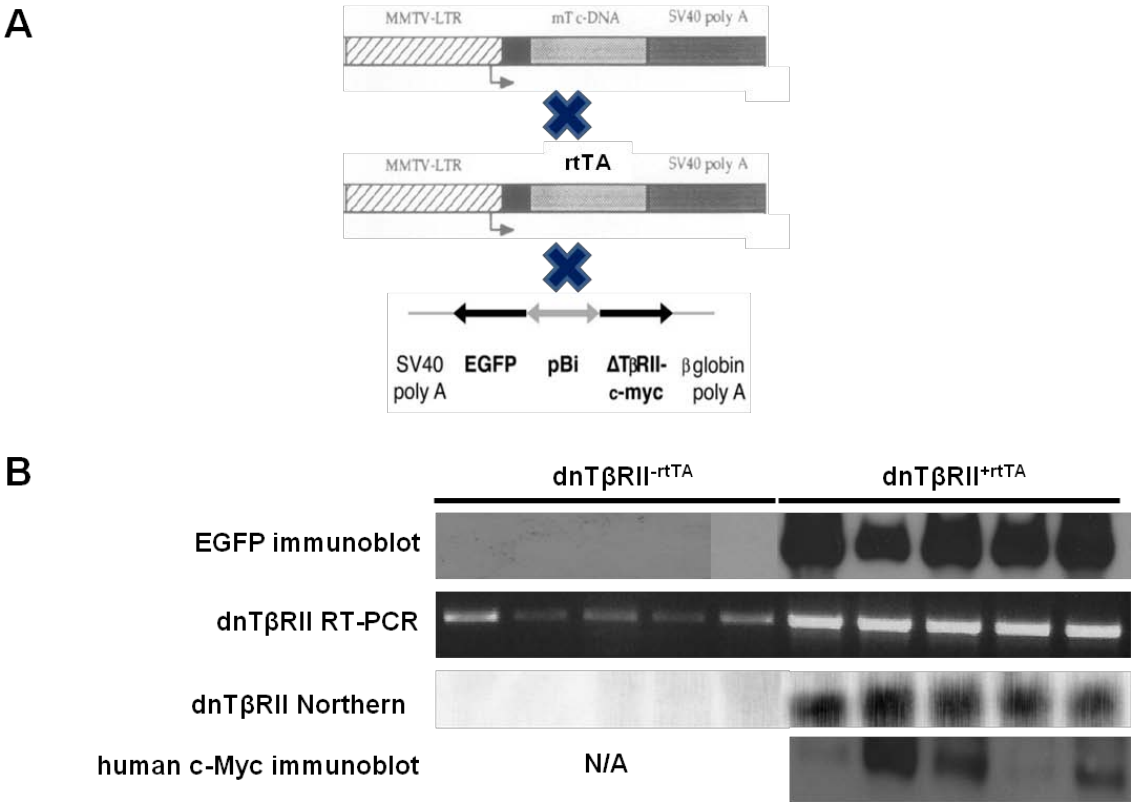

Figure 14. Dominant-negative TβRII (dnTβRII) expression in the mammary gland.

(A) Schematic of transgenic mouse model. **(B)** Expression of the dnTβRII transgene is shown for dnTβRII^{+rtTA} animals via EGFP immunoblot, RT-PCR, RNA analysis, and human c-Myc immunoblot.

Following at least 20 days of DOX treatment, expression of dnTβRII was confirmed in dnTβRII^{+rtTA} animals by dnTβRII reverse transcription PCR, RNA blotting, and EGFP and c-Myc protein expression (**Figure 14B**). At the protein level, approximately 50% of dnTβRII^{+rtTA} animals exhibited EGFP and c-Myc expression, confirming the bicistronic activity of the dnTβRII tetracycline-transactivator responsive promoter. Utilizing flow cytometry, a more sensitive detection method, all dnTβRII^{+rtTA} animals expressed EGFP (data not shown).

Tumor latency and histopathology unaffected by alteration of TGF-β signaling

In order to best exploit the temporal control of TGF-β signaling in our model, all animals were either administered DOX at least 5 days prior to tumor palpation or were treated at or after tumor palpation. In this way, this model addresses the role of TGF-β signaling in either tumorigenesis or tumor progression. Regardless of the timing of DOX administration in relation to tumor palpation, neither dnTβRII^{-rtTA} nor dnTβRII^{+rtTA} animals demonstrated significant differences in tumor onset age (**Figure 15A**). This suggests that DOX administration itself was not directly involved in tumorigenesis in our model, as has been previously suggested (Duivenvoorden et al., 2002). When directly compared with dnTβRII^{-rtTA} animals, dnTβRII^{+rtTA} animals that have attenuated TGF-β signaling had significantly delayed tumor onset when DOX-treated prior to tumor palpation (**Figure 15B**). Histopathology of dnTβRII^{-rtTA} and dnTβRII^{+rtTA} tumors revealed no difference in tumor architecture, regardless of timing of DOX treatment (**Figure 15C**, data not shown). Both animals exhibited well-differentiated, lobular structures encircled by distinct stromal features.

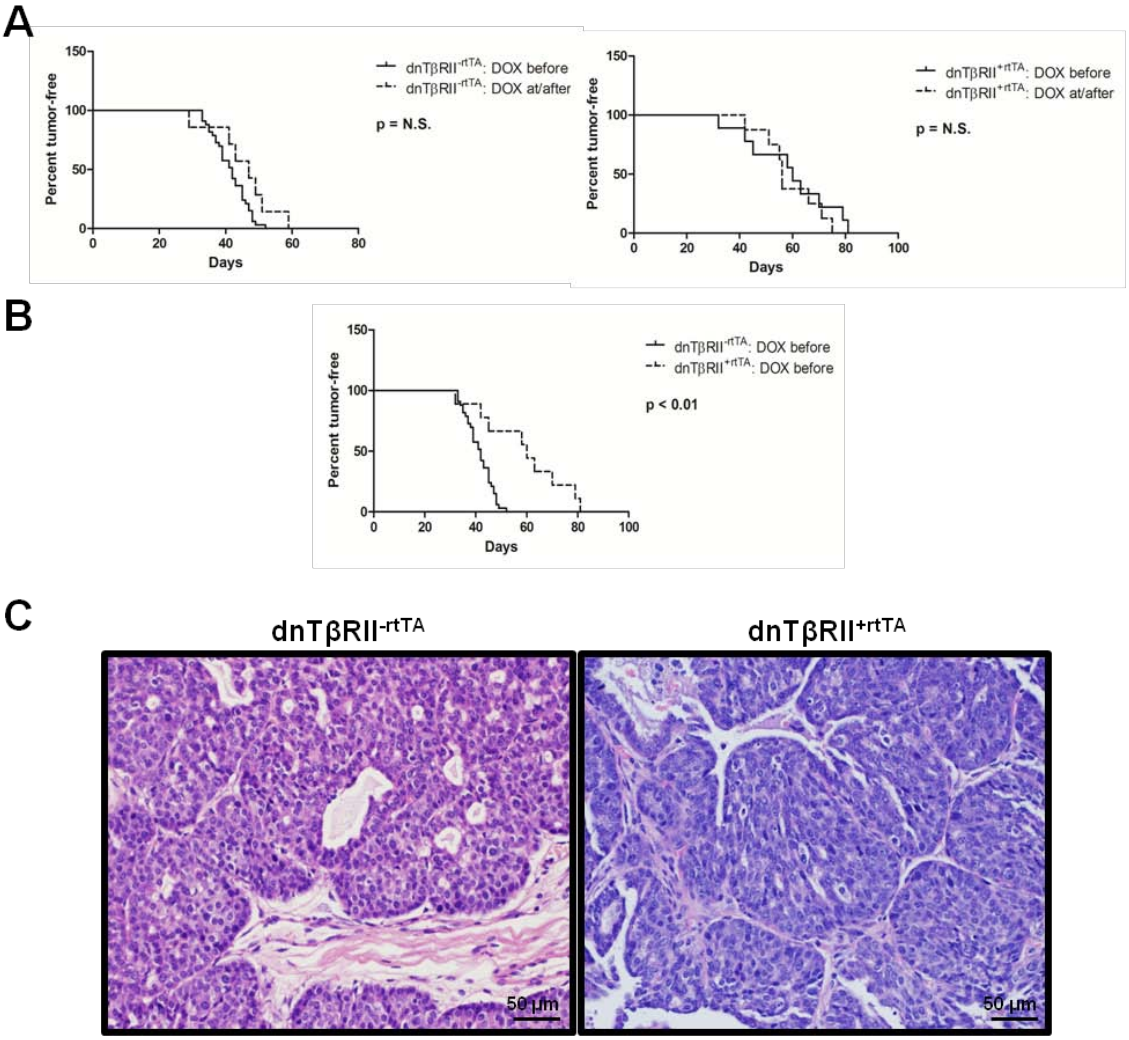

Figure 15. dnTβRII expression led to increased tumor latency.

(A) Tumor latency was compared when animals were treated with DOX prior to tumor formation or at/after tumor formation. dnTβRII^{-rtTA} (left panel) and dnTβRII^{+rtTA} (right panel) animals were analyzed. DOX was determined to have no effect on tumor latency. **(B)** Tumor latency was compared between dnTβRII^{-rtTA} and dnTβRII^{+rtTA} animals, indicating a delay in tumor onset when dnTβRII is expressed prior to tumor formation. **(C)** Overall histological comparison between dnTβRII^{-rtTA} and dnTβRII^{+rtTA} tumors.

Inhibition of TGF-β signaling prior to tumor onset increases lung metastasis

Since a significant delay in tumorigenesis was associated with attenuated TGF-β signaling, a potential connection to tumor progression was also investigated. Lungs from dnTβRII^{-rtTA} and dnTβRII^{+rtTA} tumor-bearing animals were analyzed by whole mount for the presence of lung metastases. The dnTβRII^{-rtTA} control animals had no difference in pulmonary metastases despite DOX administration prior to or at/after tumor palpation (**Figure 16A**). In contrast, dnTβRII^{+rtTA} animals trended toward increased pulmonary metastases when TGF-β signaling was attenuated prior to tumor palpation. When compared to dnTβRII^{-rtTA} lungs, dnTβRII^{+rtTA} lungs had a significantly higher frequency of lung metastases. In this way, attenuation of TGF-β signaling is not only associated with delayed tumorigenesis, but it is also associated with increased lung metastases.

Determination of active TGF-β signaling in the lungs was necessary to identify whether cells from the primary tumor could escape to a distant site of metastasis. Lungs from tumor-bearing animals were perfused, harvested, dissociated, and analyzed by flow cytometry for EGFP expression, an indication of dnTβRII expression. In day 20 primary dnTβRII^{+rtTA} tumors, 47% of tumor epithelial cells, identified as EpCAM+, express dnTβRII (**Figure 16B**). Conversely, virtually no dnTβRII+ cells were found in day 20 lungs (**Figure 16C**). This finding corresponds with previous data obtained in our lab indicating that carcinoma cells do not metastasize to the lung until day 21 post-tumor palpation (Yan et al., 2010). Additionally, the low percentage of EpCAM+ cells in the lungs (8%) indicated that the lung epithelium itself does not highly express this common epithelial marker. Any increase in EpCAM+ cells from this level was therefore attributed to an influx of carcinoma cells. This was seen in both dnTβRII^{-rtTA} and dnTβRII^{+rtTA} lungs harvested from animals bearing highly progressed tumors (**Figures 16D, 16E**). Since

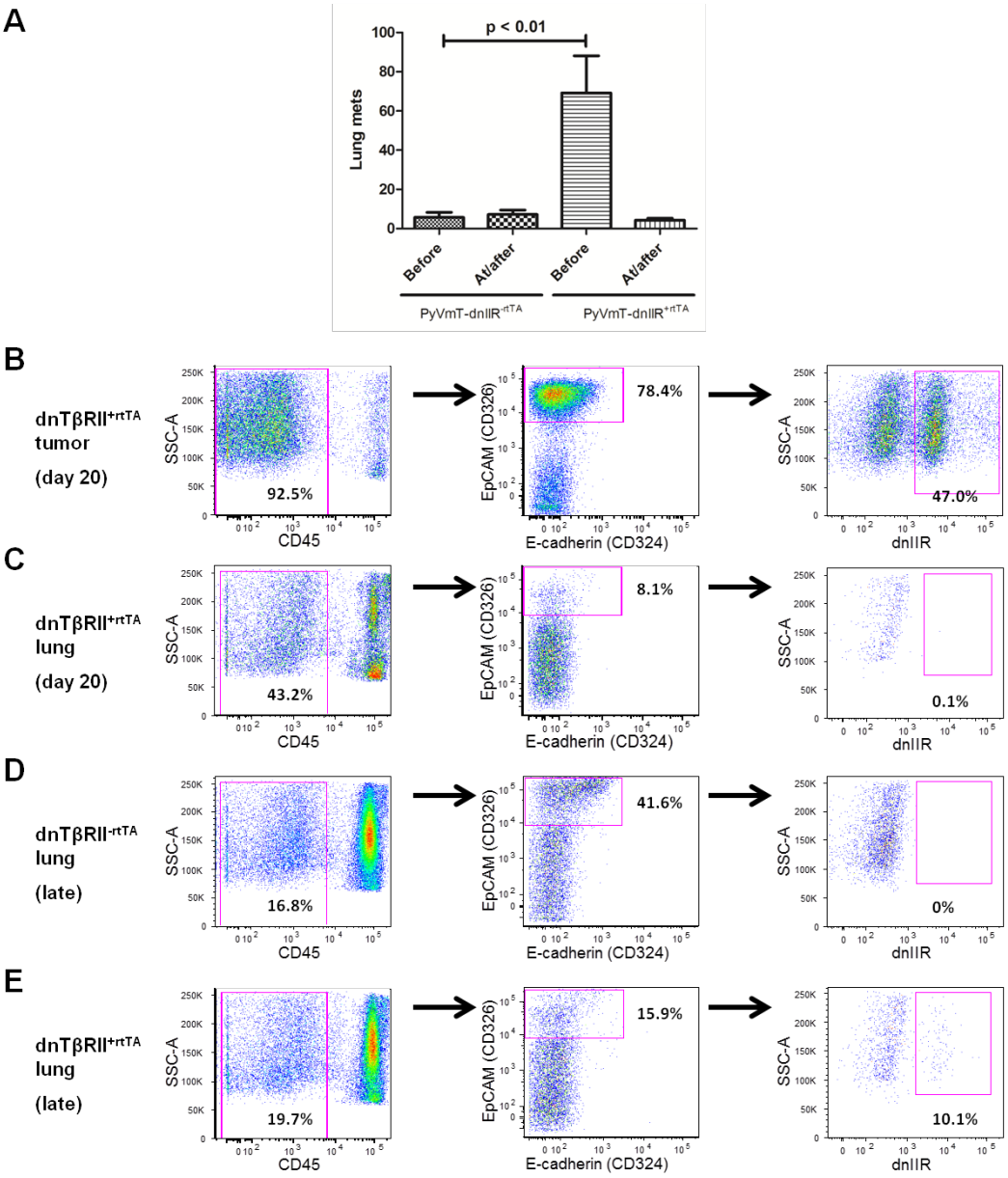

Figure 16. dnTβRII expression prior to tumor formation led to increased pulmonary metastasis.

(A) Metastases on lung whole mounts were counted for each condition listed. Animals expressing dnTβRII prior to tumor formation had increased pulmonary metastasis compared to control animals. For **B-E,** flow cytometry analysis for dnTβRII+ (EGFP+) epithelial cells was completed. **(B)** Analysis of a dnTβRII^{+rtTA} tumor 20 days post-palpation demonstrated dnTβRII epithelial expression in the primary tumor of an experimental animal. **(C)** Analysis of dnTβRII^{+rtTA} lungs from a tumor-bearing animal 20 days post-palpation revealed no lung metastasis. **(D)** Analysis of dnTβRII^{-rtTA} lungs from an animal with a highly progressed tumor confirmed that dnTβRII was not expressed in the lungs of control animals. **(E)** Analysis of dnTβRII^{+rtTA} lungs from an animal with a highly progressed tumor showed the presence of dnTβRII+ epithelial cells in the lungs.

dnTβRII expression was not found in dnTβRII^{-rtTA} lungs, controlled expression of the inducible transgene was confirmed (**Figure 16D**). This dnTβRII expression was identified in dnTβRII^{+rtTA} lungs, suggesting that primary carcinoma cells with attenuated TGF-β signaling are capable of traversing the metastatic cascade and surviving at a secondary metastatic site (**Figure 16E**). This data supports previous literature suggesting that loss of TGF-β signaling is required for re-epithelialization and colonization at metastatic sites (Giampieri et al., 2009b).

MDSC population is increased in tumors with inhibited TGF-β signaling

The immune compartment, particularly myeloid-derived suppressor cells (MDSCs) in relation to TGF-β signaling, has previously been found to play a critical role in tumor progression and metastasis (Novitskiy et al., 2011; Yang et al., 2008). The contribution of the immune system was therefore investigated as a plausible cause of increased lung metastasis in animals with attenuated TGF-β signaling prior to tumor formation. Animals were DOX-treated prior to tumor palpation and sacrificed 28 days post-palpation (**Figure 17A**). No difference in spleen immune composition, in regard to MDSCs and CD3$^+$, CD4$^+$, and CD8$^+$ T cells, was found when comparing dnTβRII^{-rtTA} and dnTβRII^{+rtTA} animals (**Figures 17B, 17C**). Although no difference in the percentages of CD45$^+$ immune cells, B and T cells, macrophages, or neutrophils were found in dnTβRII^{-rtTA} and dnTβRII^{+rtTA} tumors, there was a significant doubling of the MDSC population (**Figures 18A, 18B**). Further investigation of CD45- non-immune cells revealed no difference in quantities of epithelial (EpCAM+) or fibroblast (PDGFRα+) populations between dnTβRII^{-rtTA} and dnTβRII^{+rtTA} tumors, as well as no difference in E-cadherin expression in EpCAM+ epithelial cells (**Figure 18C**).

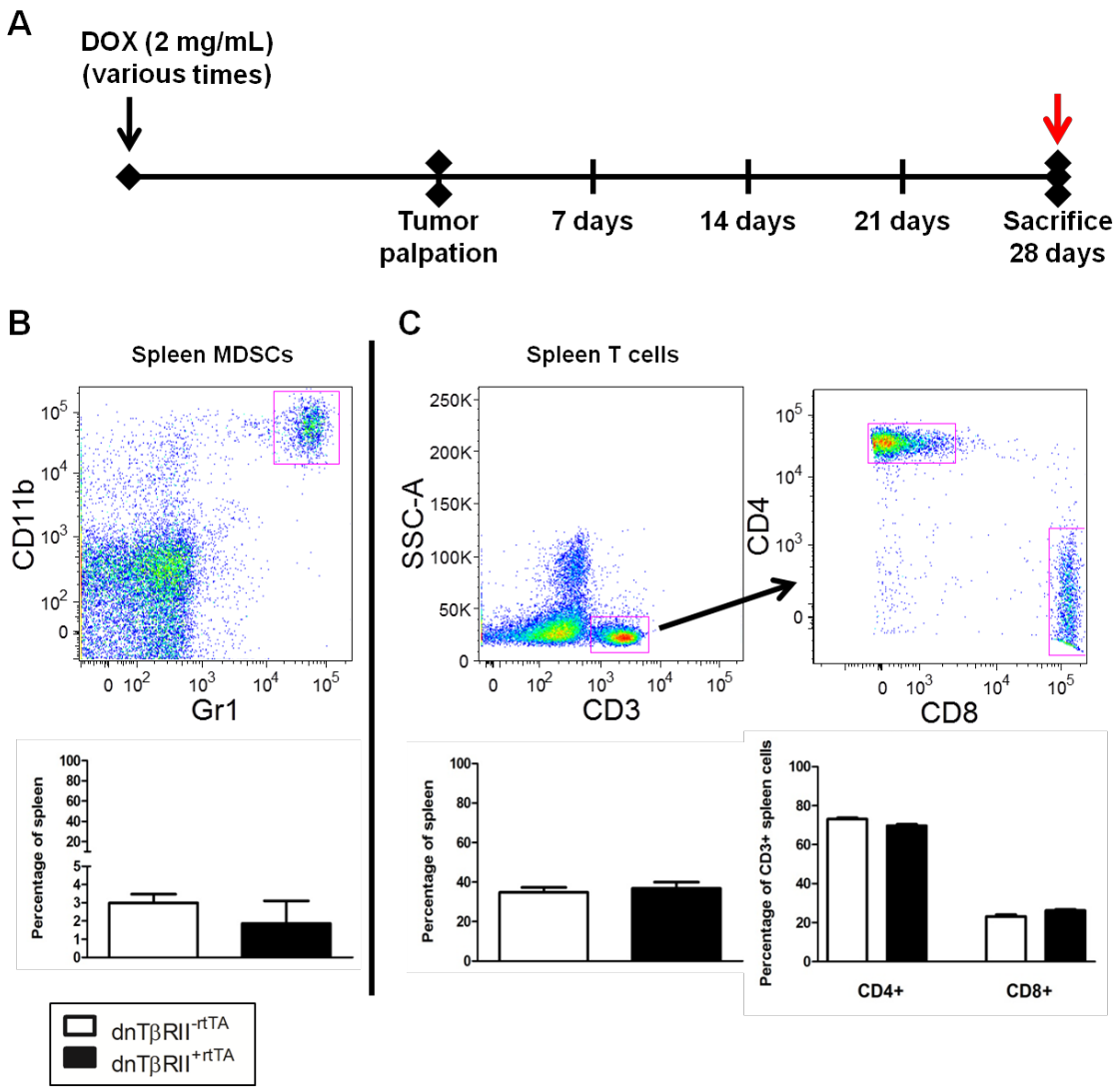

Figure 17. Flow cytometry analysis of dnTβRII^{-rtTA} and dnTβRII^{+rtTA} spleens.

(A) Schematic illustrating timing of DOX administration to animals and time of sacrifice in relation to tumor palpation. **(B-C)** Spleen MDSCs and the composition of spleen T cells were compared between dnTβRII^{-rtTA} and dnTβRII^{+rtTA} animals and demonstrated no significant differences in cell populations.

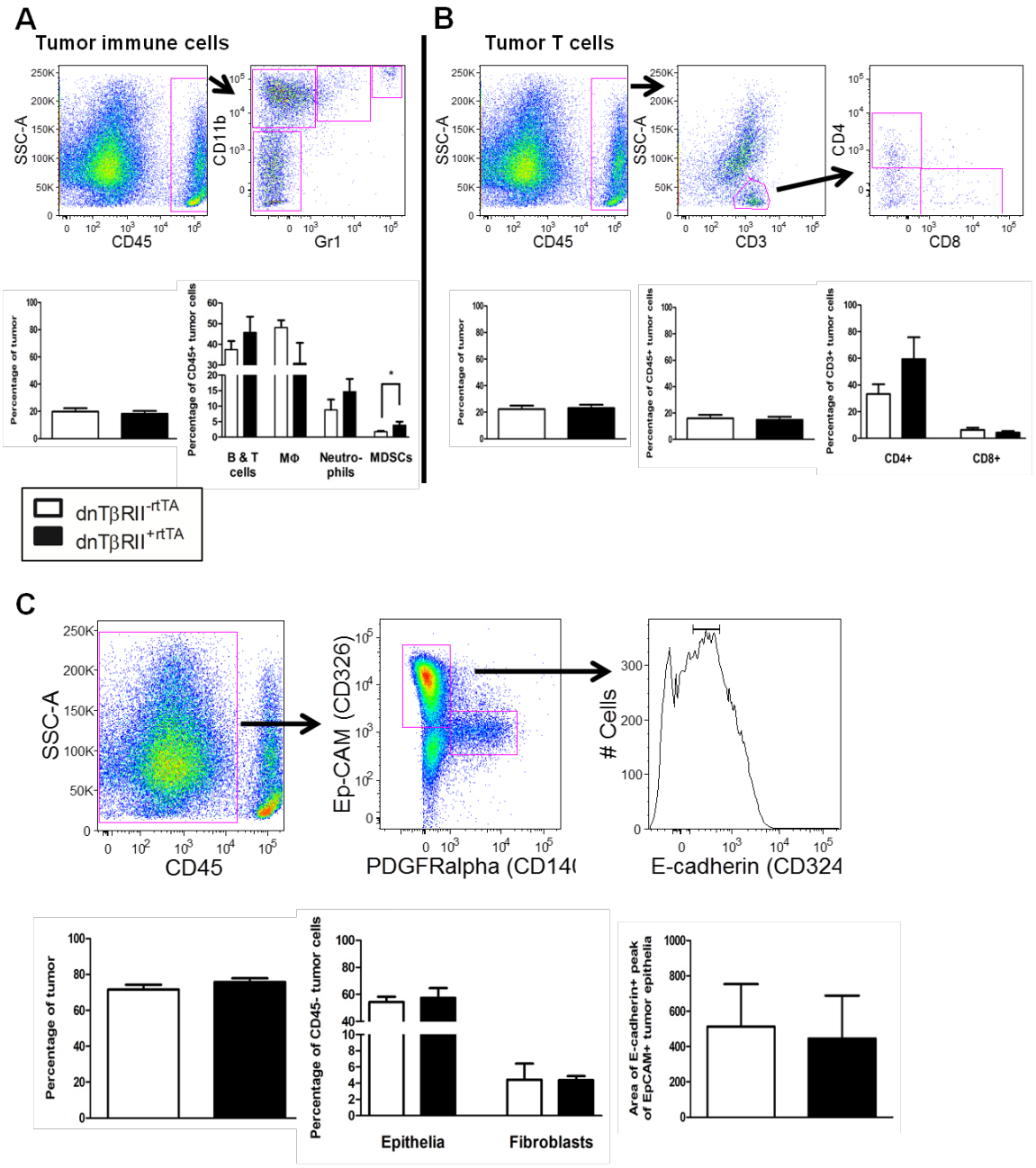

Figure 18. Flow cytometry analysis of dnTβRII^{-rtTA} and dnTβRII^{+rtTA} tumors.

(A-B) The composition of tumor immune cells and T cells were compared between dnTβRII^{-rtTA} and dnTβRII^{+rtTA} animals. An asterisk (*) denotes significance of $p<0.05$. **(C)** The composition of tumor epithelia and fibroblasts were compared between dnTβRII^{-rtTA} and dnTβRII^{+rtTA} animals. No significant differences in cell populations were noted.

Differential cytokine secretion associated with inhibited TGF-β signaling

Another potential contributor to tumor progression is an enhanced cancer cell secretome (Pavlou and Diamandis, 2010). Primary carcinoma cells from dnTβRII^{-rtTA} and dnTβRII^{+rtTA} tumors were isolated by flow sorting EpCAM+ cells. Following DOX treatment overnight, conditioned media from cells was analyzed on a cytokine array. Several targets significantly differed between dnTβRII^{-rtTA} and dnTβRII^{+rtTA} carcinoma cells (**Figures 19A, 19B, 19C**). Expression validation of these targets identified increased MCP-1/CCL2 in the same dnTβRII^{+rtTA} cells used for secretome analysis (**Figure 20**). The same expression analysis was also completed on primary tumor cells, revealing no difference in the targets investigated (data not shown). The primary tumor cells were isolated and passaged from dnTβRII^{-rtTA} and dnTβRII^{+rtTA} tumors.

Discussion

The dogmatic dichotomy of TGF-β as both a tumor suppressor and promoter has been investigated in several mouse models of cancer. Previous work from our lab has implicated TGF-β as a tumor suppressor in models of attenuation or loss of cell autonomous signaling (Bierie et al., 2008; Forrester et al., 2005). Our results substantiate this claim and demonstrate that cell autonomous TGF-β signaling must be present at the time of tumor formation in order for TGF-β-mediated tumor suppression to be effective. If TGF-β signaling is lost in epithelial cells prior to tumor formation, host defenses against tumorigenesis are attenuated and metastatic dissemination is probable. In contrast to previous models utilizing dnTβRII expression in the mammary gland, we found a decreased rate of tumor formation in animals with early attenuation of TGF-β signaling (Gorska et al., 2003). This discrepancy may be oncogene-specific

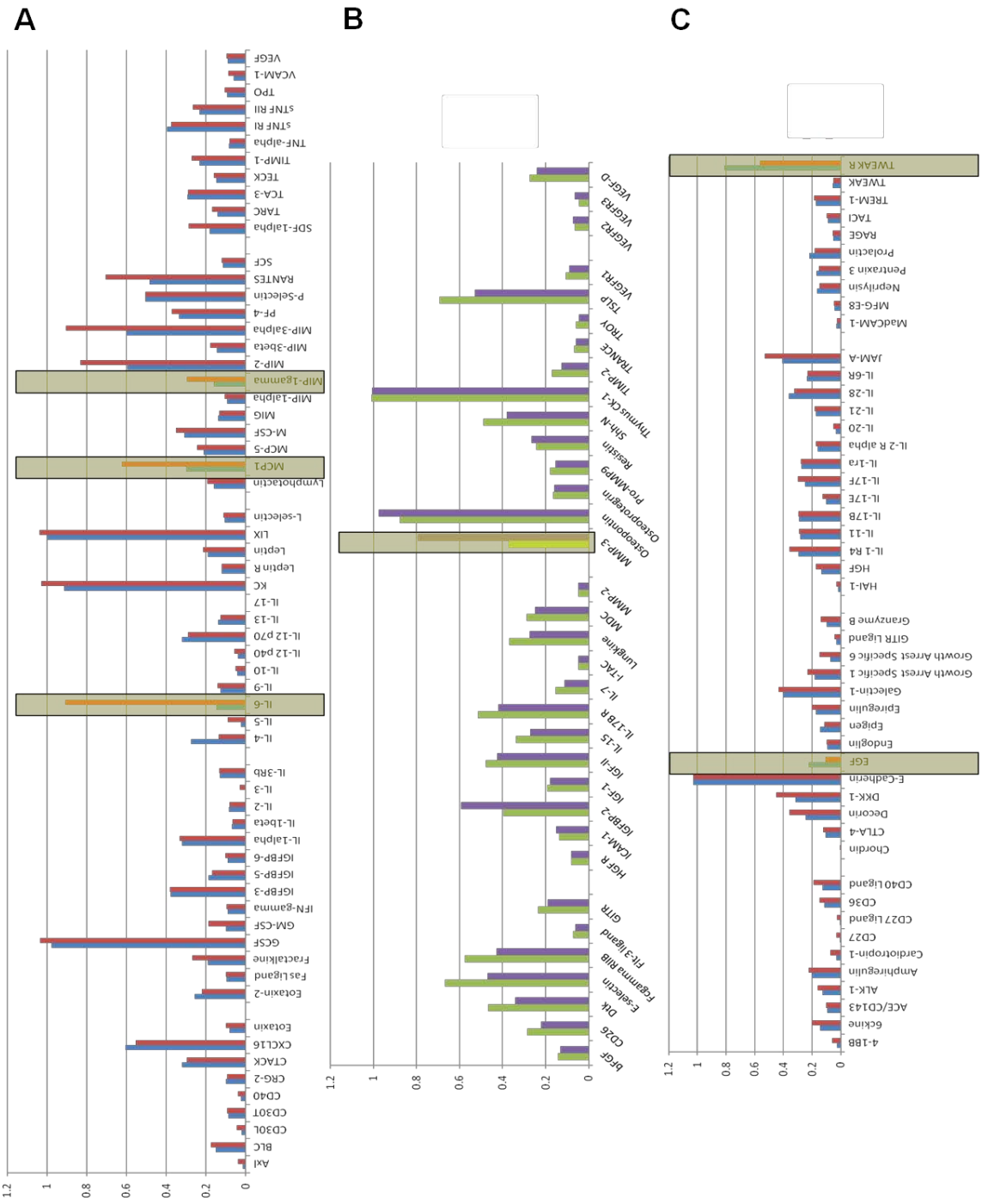

Figure 19. Cytokine array of conditioned media from dnTβRII^{-rtTA} and dnTβRII^{+rtTA} primary sorted epithelial cells.

EpCAM+ epithelial cells were sorted from three dnTβRII^{-rtTA} tumors and three dnTβRII^{+rtTA} tumors. After overnight culturing and DOX treatment, conditioned media from each tumor type was pooled for analysis on a cytokine array. Targets of interest are highlighted (yellow box), and each array **(A-C)** represents an individual blot.

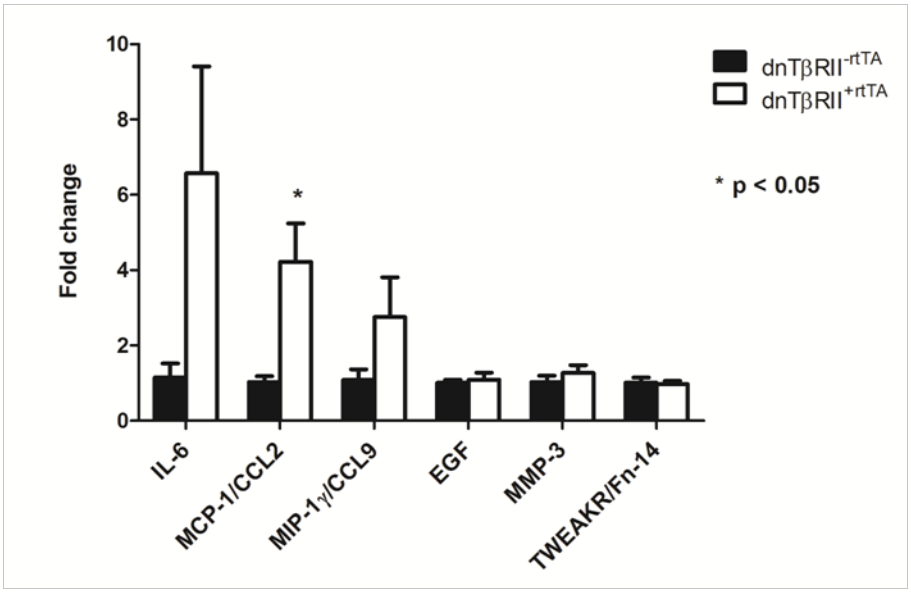

Figure 20. Cytokine array validation of target cytokines.

qPCR validation of target cytokines was completed on the primary sorted epithelial cells from which conditioned media was used for the cytokine array (Figure 19). Expression levels represent the average expression of sorted epithelial cells from three dnTβRII^{-rtTA} tumors and three dnTβRII^{+rtTA} tumors.

since our model exploited the PyVmT oncogene rather than the *TGF-α* transgene. Furthermore, our results on increased metastasis for TGF-β-attenuated carcinoma cells contradict previous reports citing decreased pulmonary metastasis when dnTβRII was co-expressed with the *c-Neu* transgene (Siegel and Massague, 2003). Our studies therefore support the role of TGF-β as a tumor suppressor but challenge the dogma of TGF-β as a metastatic promoter.

The implication of TGF-β signaling at multiple steps of the metastatic cascade has been the subject of much investigation. It is well known that TGF-β can invoke an epithelial-to-mesenchymal transition (EMT) by which carcinoma cells acquire invasive mesenchymal properties (Saitoh and Miyazawa, 2012; Taylor et al., 2011). Current dogma holds that carcinoma cells under TGF-β influence gain EMT plasticity to become single cells capable of blood-borne secondary metastasis (Giampieri et al., 2009b). Alternatively, our data suggests primary dnTβRII-expressing carcinoma cells, herein labeled with EGFP, are capable of pulmonary metastatic dissemination. This implicates attenuation of TGF-β signaling with associated cellular characteristics of collective migration and/or re-epithelialization at secondary sites.

Two potential mechanisms associated with our findings were found in the immune compartment and the cancer cell secretome. We have previously shown the importance of MDSCs to tumor progression (Novitskiy et al., 2011; Yang et al., 2008). An increased MDSC presence in early TGF-β-attenuated carcinoma cells is therefore worthy of future study. Also, identification of MCP-1/CCL2, a chemotactic cytokine for monocytes, in dnTβRII-expressing carcinoma cells is noteworthy since it has previously been reported that TGF-β increases MCP-1 expression in fibroblasts (Slavin et al., 1995). Context dependency may explain these observations seen in varying cell populations. Additionally, it has also been reported that CCL2 secretion enhances carcinoma progression when fibroblasts lack TGF-β signaling (Hembruff et

al., 2010). Further study of MCP-1/CCL2 therefore would enable mechanistic analysis of our present findings.

Our findings demonstrate a critical role for timing of TGF-β signaling during tumorigenesis and tumor progression. Early attenuation of TGF-β signaling eliminates tumor suppressive effects needed for tumor eradication, thereby substantiating the role of TGF-β as a tumor suppressor. Study of MDSC and MCP-1/CCL2 regulation of tumorigenesis and metastasis therefore deserves further consideration in this inducible dnTβRII expression model. Our model system of controlled TGF-β expression is therefore a valuable tool for identifying beneficial timing of TGF-β-targeted therapy to patients.

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

The pleiotropic functions of TGF-β in homeostasis, development, and cancer position TGF-β at the intersection of numerous cellular processes. These processes are coordinately regulated through the simultaneous interactions of multiple cell types. High levels of TGF-β ligand present in the tumor microenvironment during carcinoma progression necessitate TGF-β responsiveness of cell types other than epithelial cells. This is due to the loss of epithelial TGF-β responsiveness during tumor progression. During this progression, TGF-β has dual modalities, initially suppressing tumorigenesis while later promoting metastasis. Determining the timing of these modalities is important from a treatment perspective in order to maximize patient response to therapy. Our work presented herein furthers the current understanding of tumor-stromal interactions and offers a model for investigation of TGF-β signaling.

It is well established that epithelial cell autonomous TGF-β signaling cannot solely account for TGF-β responsiveness during tumor progression. Rather, tumor progression is shaped by the net effect of autocrine TGF-β signaling and paracrine cues emanating from stromal, endothelial, and immune cells in the tumor microenvironment (Bierie and Moses, 2006b; Massague, 2008; Taylor et al., 2011). Our work corroborates the importance of the tumor microenvironment in regulating carcinoma progression. In our murine model of breast cancer, MDSC immune populations were identified as a distinct cell population heightened in tumors with attenuated TGF-β signaling (Figure 18). MDSCs, originally identified for their

function as immune suppressors, have previously been implicated as drivers of tumor progression (Novitskiy et al., 2011; Yang et al., 2008). Concurrently, analysis of the cancer cell secretome in TGF-β-attenuated carcinoma cells proffered multiple cytokines potentially responsible for MDSC recruitment (Figures 19, 20). Mechanistic determination of these interactions should be further explored *in vitro* in primary cell lines isolated from TGF-β-attenuated and non-attenuated carcinoma cells.

Tumor microenvironmental interactions were also a predominant focus of our studies in the chicken embryo model. Our results highlight the importance of tumor-stromal interactions in tumor progression and metastasis. Sans fibroblast presence, carcinoma cells were incapable of migration (Figure 1); however, fibroblast presence stimulated an epithelial cell autonomous migratory behavior that was dependent upon TGF-β signaling (Figures 1-5). The physical presence of fibroblasts was not necessary for epithelial gene expression changes associated with this migration, as conditioned media from fibroblasts was sufficient to do so (Figure 13). In this way, identification of fibroblast-secreted factors capable of inducing migratory gene expression changes in adjacent epithelia should be explored in the future. One of these genes, Tmeff1, also deserves further investigation. Since attenuation of all Tmeff1 domains and other Nodal signaling inhibitors was observed in cells lacking TGF-β signaling, testing of Tmeff1 inhibition should be completed *in vivo* to assess potential reversal of migratory phenotype. Although fibroblasts influenced epithelial migration, reciprocity of tumor-stromal interactions was also evident. Carcinoma cells lacking TGF-β signaling increased the velocity of adjacent fibroblasts to a greater extent than did carcinoma cells with active TGF-β signaling (Figure 2). In this way, a lack of TGF-β signaling allowed carcinoma cells to respond to extrinsic stromal cues in a heightened manner and to subsequently engage in tumor-stromal communication.

A critical part to unraveling the function of TGF-β during tumorigenesis and metastatic progression lies at the interface between tumor suppressive TGF-β signaling and TGF-β promotion of metastasis. Presumably, this switch in function occurs at some instance during the metastatic cascade in which carcinoma cells escape the primary tumor, intravasate into adjacent vasculature, circulate in the bloodstream, extravasate and disseminate at secondary sites, and colonize as metastases (Valastyan and Weinberg, 2011). Navigation of this metastatic cascade therefore necessitates epithelial plasticity of carcinoma cells. Since each metastatic stage is regulated by TGF-β signaling via microenvironmental interactions, an understanding of this regulation is imperative. Our results suggest that carcinoma cells lacking TGF-β signaling have an enhanced ability to extravasate and to form metastatic loci (Figures 6, 7). This parallels our additional finding that TGF-β-attenuated carcinoma cells are better capable of pulmonary metastasis (Figure 16). Initially, these results appear counterintuitive since it has been well documented that TGF-β drives cells to undergo an epithelial-to-mesenchymal transition (EMT) (Taylor et al., 2011; Thiery, 2002). It is therefore currently thought that acquisition of EMT characteristics is requisite for invasion and vasculature entry during metastatic dissemination. Our results, however, challenge this dogma, as we have presented two separate *in vivo* models that both demonstrate enhanced metastasis of epithelial cells that have attenuation or loss of TGF-β signaling. Since we only detected these cells at the primary tumor and secondary sites of metastasis, it is still unknown whether the attenuation or loss of TGF-β signaling in our models is constant throughout the course of intravasation and extravasation. Transient and local TGF-β signaling has been previously suggested for blood borne metastasis of carcinoma cells (Giampieri et al., 2009b). It is entirely possible that transient TGF-β signaling, similar to EMT characteristics, may occur in our mouse model since signaling is only attenuated rather than lost. Investigation into this possibility should be further studied in our models.

Epithelial plasticity, as previously mentioned, is requisite for cell movement through the metastatic cascade. Given that cells must survive numerous environmental assaults during this cascade, such as stromal matrix rigidity or interstitial pressure of the vasculature during blood flow, cells must be readily adaptable during this process. In our chicken embryo model, our analysis of migration patterning demonstrates this plasticity. Fibroblast facilitation of epithelial movement generated both single cell and collective migration patterns associated with and without TGF-β signaling, respectively (Figures 1-5). Collectively migrating cells had an enhanced ability over single cells to disseminate to metastatic sites (Figures 6, 7). At the invasive forefront of collectively moving aggregates, there was evidence of leading tip cells, which serve as mechanosensors and communication conduits between the tumor milieu and the cells themselves (Figure 1). These leading cells must acquire some EMT characteristics, such as actin-rich protrusions and integrin-mediated adhesion to the extracellular matrix, in order for movement of collective aggregates to occur (Khalil and Friedl, 2010; Revenu and Gilmour, 2009; Rorth, 2007). In this way, depending upon the substrate and direction of migration, the identity of leading tip cells may change during the course of tumor progression, leading to local epithelial "melting" in which cells exhibit both epithelial and EMT-like characteristics. Evidence of both EMT and collective migratory behaviors at invasive protrusions stemming from the primary tumor demonstrates the plasticity required for cell movement and metastasis.

At the final step of the metastatic cascade, carcinoma cells must extravasate from the vasculature and colonize at a secondary site of metastasis. The concepts of mesenchymal-to-epithelial transition (MET) and re-epithelialization at these metastatic sites has emerged and remains controversial (Hugo et al., 2007; Lee et al., 2006). Cells that undergo MET are characterized by polarity markers and junctional protein expression, similar to those found in the primary tumor. Our findings in the chicken embryo model support the epithelial nature of

invasive cell movement. Our experimental metastasis assay results demonstrate that cells lacking TGF-β signaling possess an enhanced ability to extravasate, survive, and re-epithelialize at metastatic sites (Figures 6, 7). The ability to colonize at distant sites, regardless of TβRII expression and cell quantity, is supporting evidence for MET. Analysis of metastasis in our mouse model also shows that cells with attenuated TGF-β signaling are capable of lung metastasis. Since these cells have low levels of TGF-β signaling, it is presumed that these cells are able to form epithelial junctions and maintain polarity. Further study of lung histological sections would address this question and help determine what percentage of TGF-β-attenuated carcinoma cells constitute the entirety of visible lung metastases. It is possible that out of all disseminated carcinoma cells, only a few TGF-β-attenuated carcinoma cells are required for any lung colonization.

Elucidating the timing and corresponding function of TGF-β signaling during tumorigenesis and tumor progression is a subject of current investigation. We have presented a mouse model that exploits attenuation of TGF-β signaling at different time points during tumor formation and progression to address these questions. Although previous models have ablated TβRII to study epithelial cell autonomous behavior (Bierie et al., 2008; Forrester et al., 2005), human data suggests that *TGFBR2* is genetically stable in the majority of breast cancer (Levy and Hill, 2006). Deregulation of TβRII expression, however, has been frequently reported. Low levels of TβRII expression correlated with an increased breast cancer risk in women with breast hyperplastic regions lacking atypia, while loss of TβRII expression correlated with increased tumor grade (Gobbi et al., 2000; Gobbi et al., 1999). Thus, a more relevant model for study of TGF-β signaling utilizes attenuation of the signaling pathway since most human cancers demonstrate loss of TGF-β signaling during tumor progression. Our mouse model replicates the attenuated signaling observed in humans through the use of inducible dnTβRII expression.

Past mouse models of breast cancer utilizing dnTβRII expression resulted in lobular alveolar hyperplasia and an increased rate of tumor formation in conjunction with a *TGF-α* transgene; however, decreased pulmonary metastasis resulted when dnTβRII was expressed along with a *c-Neu* transgene (Gorska et al., 2003; Siegel et al., 2003). Our results challenge these previous reports in that expression of dnTβRII in conjunction with PyVmT in our model both increased tumor latency and potentiated metastasis. Our findings therefore support the role of TGF-β as a tumor suppressor but do not provide credence for TGF-β functioning as a metastatic promoter. One distinct advantage of this mouse model is the ability to activate dnTβRII expression at any desired time. In our studies, dnTβRII expression was only investigated prior to and at/after tumor palpation (Figure 15). Manipulation of the timing of dnTβRII expression therefore deserves future investigation. For instance, since early and continued attenuation of epithelial TGF-β signaling led to increased pulmonary metastasis, reversal back to active TGF-β signaling after tumor formation might affect metastatic potential. Additionally, the ability to turn on/off dnTβRII expression also frames future studies on investigation of transient and long-term effects of attenuated epithelial TGF-β signaling. All of these studies are important to the timing of TGF-β-based therapeutic administration. Correlation between these therapies and the stage of tumor progression at which they should be administered is the subject of current studies.

Summary and concluding remarks

Results presented herein implicate carcinoma cell autonomous TGF-β signaling as a critical mediator of the metastatic cascade. Loss of this autonomous signaling was associated with increased invasive potential and metastatic propensity. In the chicken embryo model, this

epithelial behavior was concomitant with paracrine signaling cues emanating from adjacent stromal fibroblasts in the tumor milieu. This stromal influence stimulated either single cell or collective migration of cells with and without TGF-β signaling, respectively. Additionally, detection of collectively invading tumor cells at metastatic sites illustrates the plasticity required for cells to navigate the metastatic cascade and to re-epithelialize during formation of metastatic foci. In our attenuated TGF-β signaling mouse model, we have shown that cells with attenuated signaling are also capable of metastasis. Results obtained in our chicken embryo and murine studies therefore augment our current understanding of tumor-stromal interactions and the biphasic nature of TGF-β. The cell type- and context-dependent nature of TGF-β signaling in the microenvironment is complex, as evidenced in our studies, and should be further elucidated. As such, a more comprehensive understanding of cell type- and context-dependent TGF-β signaling is therefore needed in order to improve the timing and choice of therapy regimens for breast cancer patients. We believe that our presented findings help to unravel the complex web of TGF-β signaling in carcinoma progression and metastasis.

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