TRANSFORMING GROWTH FACTOR BETA-INDUCED TYROSINE PHOSPHORYLATION OF SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3 AND CELLULAR INVASION IS MEDIATED BY INTERLEUKIN-6 SECRETION IN BREAST CANCER CELL LINES

By

NICOLE N. TEOH PARKER

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To my family and friends, who always support me;
To my husband, who is my best friend and so much more

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LIST OF ABBREVIATIONS

ADAM	A Disintegrin And Metalloprotease
ALK	Activin-Like Kinase
ATX	Autotaxin
bFGF	basic Fibroblast Growth Factor
BMP	Bone Morphogenic Protein
cEBP	CCAAT Enhancer Binding Protein
CK	Cytokeratin
CREB	Ca^{2+}/cAMP-Response Binding Element
CM	Conditioned Medium
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
GF	Growth Factor
gp130	Signal transducing cytokine receptor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HGF	Hepatocyte Growth Factor
Id1, Id2	Inhibitor of Differentiation 1, 2
IL-6	Interleukin-6
IL-6R	Interleukin-6 Receptor

IL-10	Interleukin 10
Jak	Janus kinase
LAP	Liver-enriched transcriptional Activator Protein
LIF	Leukemia Inhibitory Factor
LIFR	LIF Receptor
LPA	Lysophosphatidic Acid
m67-Luciferase	Reporter construct containing 4 STAT3 binding (m67) sites
mIL-6-RFP	mouse IL-6 Receptor Fusion Protein Inhibitor
MMP	Matrix Metalloproteinase
NF-κβ	Nuclear Factor Kappa Beta
PA	Phosphatidic Acid
Pai-1	Plasminogen Activator Inhibitor-type I
PDGF/R	Platelet-Derived Growth Factor (Receptor)
PIAS	Protein Inhibitor of Activated STATs
PLD	Phospholipase D
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
SH2	Src Homology 2
SHP-2	Src Homology 2-containing tyrosine phosphatase
sIL-6Rα	soluble Interleukin-6 Receptor α
siRNA	small interfering ribonucleic acid
SIS3	Specific Inhibitor of Smad3
SMA	Smooth Muscle Actin
SOCS	Suppressor of Cytokine Signaling
STAT3	Signal Transducer and Activator of Transcription 3

STAT3-C	Constitutively Activated STAT3
TβRI (or II)	Transforming Growth Factor β Receptor I (or II)
TβRKI	Transforming Growth Factor β Receptor I Kinase Inhibitor
TDF/TDMF	Tumor-Derived Fibroblasts/Myofibroblasts
TGFα	Transforming Growth Factor α
TGFβ	Transforming Growth Factor β
TIMP	Tissue Inhibitor of MetalloProteases
TMA	Tissue Microarray
TNFα	Tumor Necrosis Factor α
VEGF	Vascular Endothelial Growth Factor

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By

Nicole N. Teoh Parker

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A hallmark of breast cancers is the overexpression or constitutive activation of various oncoproteins. Two examples are the proteins Signal Transducer and Activator of Transcription 3 (STAT3) and Transforming Growth Factor β (TGFβ). Although each of these signaling molecules and their respective pathways have been implicated in cancer development, the mechanism of cross-signaling between these pathways has not been established in the cancer setting. We hypothesize that cross-talk between the TGFβ and STAT3 pathways contributes to cancer invasiveness through an autocrine signaling loop.

Preliminary data in nontransformed mouse mammary NMuMG cells indicate that exogenous TGFβ treatment results in phosphorylation of STAT3 on its activating Tyrosine (705) site. Strikingly, this effect is observed only after several hours of TGFβ treatment and appears to be mediated by the cytokine Interleukin-6 (IL-6). TGFβ induced IL-6 mRNA upregulation occurs concomitantly with TGFβ-stimulated STAT3 tyrosine phosphorylation. Blockade of IL-6 function with a mouse IL-6 receptor fusion protein abrogates this effect, thereby implicating IL-6 as the factor responsible for TGFβ-stimulated STAT3 tyrosine phosphorylation in NMuMG cells. TGFβ confers an invasive phenotype on NMuMG and Mv1Lu cells that is IL-6 dependent.

Examination of this mechanism in human breast cancer cell lines suggests that TGFβ-mediated secretion of IL-6 is responsible for the constitutive STAT3 tyrosine phosphorylation observed in many breast cancer cell lines. The invasive human MDA-MB-231 basal-like breast cancer cell line exhibits TGFβ and IL-6 dependent invasion, while the noninvasive human MDA-MB-361 luminal breast cancer cell line is capable of attaining IL-6-dependent cellular invasiveness in response to TGFβ treatment.

CHAPTER 1
INTRODUCTION

Breast Cancer: A Continuing Disease

Breast Cancer Incidence

Among women breast cancer is the most frequently diagnosed cancer and is the second leading cause of cancer-related deaths, with an estimated 27% of new cancer cases in women in the United States in 2009 (1). Risk factors include diet (2), BRCA1/2 mutations (3), and drastic changes in body weight (4). Carcinogenesis, or the development of cancers from epithelial tissues in the body, results in the development of bulky tumor masses. A hallmark of cancers is the overexpression or hyperactivation of various oncogenic proteins. Although cancer etiologies differ widely, a successful cancer must possess the following abilities (5): 1) Heightened cell cycling and reproductive ability, with a loss of cell cycle checkpoints and regulation, and development of autonomous growth factor production; 2) Anti-apoptotic behavior; 3) Increase in angiogenesis and the associated 4) Invasive and metastatic ability. Common chemotherapies concentrate on eliminating tumor burden and therefore reducing the cell population that possesses metastatic ability. Strikingly, a majority of cancer patients succumb not to primary cancers but to metastatic lesions, which are more difficult to detect and eliminate (1).

Despite numerous advances in chemotherapeutics, the mechanisms involved in tumorigenesis are still being characterized. The basis for the development of new therapies relies on targeting receptors or other oncogenic proteins that are overactivated in cancers. However, the observation that there is oncogenic overactivation or constitutive activation of proteins in certain cancer types is correlative at best, and these dysregulations are not necessarily the driving event behind the development of a cancer. Therefore, therapies targeting proteins that help maintain a cancer's survival, but not necessarily its progression per se, can augment

anticancer regimens. However, such strategies may be regarded as insufficient due to a potential inability to inhibit the molecular forces that drive tumorigenesis. A critical part of breast cancer research relies not only on the development of specific agents to target cancers, but also on basic research that elucidates the mechanisms of carcinogenesis. In so doing, the particular etiology of a cancer may be uncovered and a potentially preventative or inhibitory pharmacological approach can be taken. For example, treatment with an anti-oncogenic therapy in early phases of carcinogenesis may prevent the cancer's development into a big bulky mass, and therefore halt its invasion and metastatic ability, thereby improving the patient's prognosis.

TGFβ and STAT3 Each Exhibit Pro- and Anti-Tumorigenic Roles in Cancer

Two examples of oncogenic proteins are the growth factor Transforming Growth Factor β (TGFβ) and Signal Transducer and Activator of Transcription 3 (STAT3). The transcription factor STAT3 is required for Src-mediated transformation (6, 7). Overexpression of STAT3 has been observed in many cancers (8) and more recently has been demonstrated to play a positive role in tamoxifen response (9) and to correlate inversely with overall patient survival (10, 11). Conversely, STAT3 contributes to other confounding effects such as chemoresistance in breast cancers (12). STAT3, therefore, is a factor whose precise role in breast cancer is still being elucidated.

Similarly, TGFβ is a factor that has an elusive role in cancer. Expression of TGFβ protein correlates with shorter disease-free survival in patients with early stage primary breast cancer (13). TGFβ can cause estrogen insensitivity, leading to refractory responses to such hormone-based breast chemotherapies as tamoxifen (14). Although TGFβ causes growth inhibition in the MDA-MB-231 breast cancer cell line (15), it is also required for metastasis of this same cell line into bone (16). Many studies have demonstrated the significance of serum

TGFβ concentrations, as serum may be obtained with little invasiveness to the patient. Higher TGFβ serum concentrations were found to correlate with decreased survival in breast cancer patients (17, 18) and in breast cancer patients expressing a polymorphism in the Her2 gene (19).

Transforming Growth Factor β

Mechanisms of TGFβ Signaling

The cytokine TGFβ was first identified as a molecule with mitogenic properties, but this effect was later attributed to a different factor, TGFα, which is structurally and functionally similar to Epidermal Growth Factor (20,21). TGFα was responsible for the pro-growth effects observed previously, while TGFβ was shown to be growth inhibitory in many systems (22).

The TGFβ molecule belongs to a larger group of ligands termed the TGFβ Superfamily (23). This family includes many molecules, notably the Bone Morphogenic Proteins (BMPs), that are involved in development, tissue differentiation, and some cancers (24-27). The BMPs and other molecules signal in a manner analogous to TGFβ signaling, but interact with a separate group of BMP-specific receptors and downstream effectors (28). TGFβ-mediated signaling occurs through the interaction of TGFβ with the Activin-Like Kinase (ALK5) protein (TGFβ Type I receptor, or TβRI), which heterodimerizes with the Type II TGFβ receptor (TβRII) (29-32). These receptor-TGFβ oligomers associate in higher-order structures, forming a complex with a stoichiometric TGFβ-TβRI-TβRII ratio of 2 : 2 : 2 (31, 33). The TGFβ Type I (TβRI) and Type II (TβRII) Receptors are both required for intact TGFβ ligand-stimulated signaling (34).

The TGFβ-TβRI-TβRII complex results in TβRII transactivation by TβRI (31), whereupon the Smad proteins are recruited to the receptors (35) (Figure 1-1). The TGFβ receptor-associated Smads are Smads 2 and 3 (36). These Smads are recruited to activated receptor dimers, where they are phosphorylated on key serine/threonine sites (37).

Phosphorylated Smads diffuse away from the receptor dimers and heterotrimerize with the coactivating (Co-Smad) Smad4 protein (38).

After Smad heterotrimerization the complex translocates into the nucleus, where it can interact with cofactors such as p300 (39) and with Histone H3 (40) and alter the transcription of target genes (41), the promoters of which typically contain CAGA repeats (42). Examples of TGFβ-Smad target genes include the Epidermal Growth Factor Receptor (20, 41), Survivin (43), Myc (44), p21 (45), and Plasminogen Activator Inhibitor-type 1 (Pai-1) (46). Other examples of cancer-related genes that are regulated by TGFβ include Matrix Metalloproteinases (MMPs) (47, 48), Tissue Inhibitors of MMPs (TIMPs) (49, 50), and Vascular Endothelial Growth Factor (VEGF) (51, 52). Strikingly, this diverse array of genes falls into both anti- and pro-tumorigenic categories, which underscores the paradox of TGFβ in cancer [for reviews, see (53-55)].

The Paradoxical Role of TGFβ in Carcinogenesis

As previously mentioned TGFβ is a cytokine that induces both tumor suppressive and oncogenic downstream functions. In nontransformed epithelial cell lines this cytokine induces growth arrest (56) and the process of Epithelial to Mesenchymal Transition (EMT) (57, 58), although the latter event only appears to occur in some cell lines and not others (59).

Key members of the TGFβ signaling pathway are mutated, downregulated, or absent in various cancers. Examples are the Smads (25, 60, 61), the TGFβ receptors (62, 63), and TGFβ itself (64). The Type III TGFβ receptor (TβRIII) can act as a tumor suppressive molecule, binding and sequestering TGFβ Type I and Type II receptors and preventing their stimulation by TGFβ (65-70). The Type 1 isoform of the TGFβ ligand is present in the serum of breast cancer patients with advanced disease (71) as compared to normal patients. TGFβ is overexpressed (72) and secreted by multiple cancer cell lines (72, 73) and tumor-derived cells, including fibroblasts

(74), and is necessary for cross-talk between tumor-infiltrated fibroblasts and neoplastic cells (75).

However, the exact role of TGFβ in carcinogenesis remains ill-defined, due in large part to a "TGFβ Switch" that signifies the point at which TGFβ ceases tumor suppressive functions and becomes an oncogenic factor [(76, 77); reviewed in (78)]. Further complicating the issue is the fact that the observation of TGFβ present in cancers derived from patients is only correlative data: accrued mutations or changes in gene expression are observations made only after the tumor is formed, and no definite conclusions may be drawn about the tumor's actual disease pathology. Therefore, understanding how TGFβ contributes to carcinogenesis will aid the discovery of more efficacious treatments in the clinic.

TGFβ and Cancer: Diagnostic and Therapeutic Implications

TGFβ is a factor displaying both anti- and pro-tumorigenic downstream effects (79), and multiple members of the canonical TGFβ signaling pathway are mutated or dysregulated in various cancers (61, 80). However, despite seemingly contradictory roles in cancer TGFβ functions in late carcinogenesis as a pro-tumorigenic factor, and its levels in serum are elevated in breast cancer patients whose disease is further progressed (71, 81) as well as in patients suffering other cancer types (82, 83). TGFβ is required for cancer cell invasiveness (57, 84) and inhibition of TGFβ signaling correlates with breast cancer patient outcome (85). The development of novel TGFβ inhibitors has formed a solid basis for identification of new cancer therapies (86, 87). Many approaches have been taken to abrogate TGFβ in various types of cancers (87-98), some of which have been indirect. Some chemotherapies that are already clinically available but whose mechanisms were not well characterized have more recently been demonstrated to act through inhibition of the TGFβ pathway (90, 96, 97). This "post-clinical"

elucidation of cytotoxic chemotherapeutic mechanisms holds promise for further characterization of other therapies that are efficacious in the clinic but whose mechanisms are not yet well understood.

Signal Transducer and Activator of Transcription 3

Cytokine Induced STAT3 Signaling and Mechanisms of STAT3 Activation

STAT3 is a latent cytoplasmic transcription factor and, upon phosphorylation of its activating Tyrosine (705) site, forms dimers with a tyrosine phosphorylated STAT3 or STAT1 molecule and translocates into the nucleus (99). STAT3 tyrosine phosphorylation can be induced by many cytokines and growth factors including Interleukin-6 (IL-6) (100), IL-10 (101), Leukemia Inhibitory Factor (LIF) (102, 103), Oncostatin M (104), Platelet-Derived Growth Factor (PDGF) (105), basic Fibroblast Growth Factor (bFGF) (102), Epidermal Growth Factor (20, 106, 107), and by intracellular kinases such as Src (8) and Ras (108).

STAT3 dimers then bind the promoters of target genes such as p21 and Cyclin D1 (109), MMP-9 (110), and the oncogene Pim1 (111). Many target genes of both activated and nonphosphorylated STAT3 have been identified that are necessary for such downstream functions as wound healing (112) and cancer (113), respectively. Significantly, STAT3 can either activate or repress its target genes (114) through its interactions with other transcription factors such as JunB (115), or with cofactors such as p300/CREB (116). Further, STAT3 upregulates other genes necessary for additional cancer processes such as proliferation, angiogenesis and cell survival (117, 118).

Phosphorylation of STAT3 on Serine (727): Transcriptional significance

There is a large body of literature examining the Serine (727) site of STAT3 and its effect on STAT3 transcriptional activity (119-123). Some groups have demonstrated that phosphorylation of this site is required for maximal STAT3 transcriptional activity (120), others

have shown that this phosphosite represses STAT3 transcription (124, 125), and still more have suggested that the Ser (727) site possesses no transcriptional significance (119, 122). While all of these studies and data are convincing, it is clear that regulation of the two STAT3 phosphosites—and in particular the STAT3 Ser (727) site—is a complex and cell specific feature. The significance of this site lies beyond the scope of this study. Therefore, we will not provide detailed analysis of STAT3 Ser (727) signaling, although we have examined the regulation of this phosphorylation site in the course of this study.

Regulation of STAT3 Signaling

There are several levels of STAT3 regulation that operate mainly through STAT3 dephosphorylation. STAT3 tyrosine phosphorylation is attenuated by the Src Homology 2-Containing (SHP) tyrosine phosphatases in the nucleus (126), while the Suppressor of Cytokine Signaling (SOCS) proteins regulate STAT3 activity by binding the gp130 receptor and thereby preventing STAT3 occupancy of the same region (127). The Protein Inhibitor of Activated STATs (PIAS) proteins are responsible for dephosphorylating STAT3, rendering STAT3 monomers favorable for exportin binding, nuclear export and future reactivation (128-130). One group has shown that STAT3 binding domains in the SOCS3 pathway are essential for STAT3-induced upregulation of SOCS3, implying autoinhibition of IL-6 induced STAT3 signaling under normal circumstances (131). Additional spatiotemporal regulation of STAT3 can involve proteolytic processing of STAT3 protein (132), alternative splicing to obtain the STAT3-alpha (wild-type) and STAT3-beta (truncated) isoforms (133), or p300/CREB-binding protein (CBP)-mediated acetylation of a key lysine residue for proper nuclear export (134), in addition to SUMO-mediated ubiquitination and degradation of the STAT inhibitory phosphatases SOCS and PIAS (130). The STAT3β isoform is naturally present, and acts as a dominant-negative form of STAT3 by binding and inhibiting phosphorylated STAT3 activity or by occupying the STAT3

promoter binding sites and preventing dimer-mediated transcription (135). STAT3 proteins, whether nonphosphorylated or tyrosine phosphorylated, reside in membrane lipid rafts before translocation into the nucleus (136), and activated STAT3 exhibits a higher rate of nucleocytoplasmic shuttling than non tyrosine-phosphorylated STAT3 (137). Significantly, the coiled-coil domain of STAT3 is required for nuclear translocation as well as nuclear retention (138, 139). STAT3 transport to the nucleus by endocytosis is another physical level of regulation that may be disrupted by endocytic inhibitors (140). Once in the nucleus, STAT3 dimers must bind proper cofactors and coactivators such as p300/CBP (134) to properly transactivate target genes. Several studies suggest that STAT3 transactivation of sites such as the LAP/cEBPbeta promoter (141) require STAT3 tethering to nearby DNA through cofactor activation and protein complex formation.

IL-6 Signaling: Normal Cytokine Signaling Versus sIL-6R Trans-Signaling

Interleukin-6 (IL-6) cytokine signaling (Figure 1-2) occurs when IL-6 ligand binds to the IL-6 receptor (IL-6R), which then heterodimerizes with the signal transducing receptor, gp130 (142). Upon dimerization the receptors become transactivated at key tyrosine sites, to which the soluble Jak kinases bind (143). These activated Jaks then phosphorylate STATs at the receptor. After phosphorylation on key tyrosine sites the STATs diffuse away from the receptor and are free to dimerize and translocate into the nucleus (99). However, this model is only one part of the IL-6 signaling pathway. Additional stimulation can also occur through a trans-signaling mechanism, in which a soluble form of the IL-6R (sIL-6R) is present in the extracellular milieu of the cell (144). The sIL-6R binds membrane-bound gp130 and potently activates gp130-mediated signaling (145-151) (Figure 1-3). Interestingly, the extracellular domains D1-D3 of gp130 naturally occur as soluble receptors (152-155), but potently inhibit IL-6R-gp130 mediated signaling (156, 157).

The sIL-6R, which is the extracellular portion of the IL-6R, can be obtained by alternative splicing or by proteolytic cleavage. The ADAMs, or A Disintegrin and Metalloproteases (158, 159), cleave proteins such as the IL-6R with intrinsic MMP-like activity (159). In particular, ADAMs 10 and 17 (158) have been demonstrated to be important for cleavage and, therefore, activation of IL-6R and other receptors and ligands such as TNF-α (160) and Heparin-Binding Epidermal Growth Factor (HB-EGF) (161-163).

STAT3 and Cancer

STAT3 has varied downstream effects on the various stages of carcinogenesis and metastasis. STAT3 has been demonstrated to be essential for Src-mediated transformation (6, 7) and IL-6 mediated transformation (164), and is overexpressed in many cancers (8). Exogenous expression of a constitutively activated form of STAT3 revealed that this transcription factor is required for the development of skin cancer (165). STAT3 largely contributes to upregulation of genes that are required for cancer processes such as proliferation and evasion of apoptosis (117). In addition, STAT3 inhibits p53 function and can downregulate p53 at the gene transcription level, leading to abrogation of p53-mediated DNA repair mechanisms and cell cycle arrest (166) (Figure 1-4).

Further, as such a promiscuous agent in the cancer process, STAT3 appears to have a role in every step of carcinogenesis, and correlates with histologic grade in mammary cancers (167) and invasive potential in prostate cancers (168). Blockade of STAT3 in certain systems can not only restore apoptosis but can also induce cell cycle arrest and reduce cellular invasion (169) and cause growth suppression (170). Breast cancer metastasis requires only STAT3 phosphorylation on Tyr (705) and not on Ser (727) (122). STAT3 is important for maintenance of telomerase activity in prostate cancer cells (171) and, perhaps most clinically relevant, STAT3 is essential for hypoxia-induced angiogenesis (172-176) and for invasion and metastasis (177). IL-6

contributes to multi-drug resistance (178), androgen-independent prostate cancer growth (179), is expressed at high levels in basal-like breast cancers (180), and is abundant in the serum of breast cancer patients (181). Further, inhibition of IL-6 function inhibits breast cancer invasiveness (182-184).

Other upstream molecules besides IL-6 can drive STAT3 oncogenic activity. In particular, cross-talk with other signaling pathways such as the Epidermal Growth Factor (20) pathway can strongly induce STAT3 activity (185, 186). EGF receptor (20) mediated STAT3 tyrosine phosphorylation contributes to cell survival, or evasion of apoptosis, in head and neck cancers (187), and also induces autocrine growth signaling in small cell lung carcinoma cells through STAT3-dependent Leukemia Inhibitory Factor (LIF) secretion (188). LIF expression also contributes to murine mammary carcinomas (189), while EGF signaling can stimulate activation of the soluble Src kinase (107).

Diagnostic and Therapeutic Implications

STAT3 has gained interest as a drug target due to its overexpression and constitutive activation in a large variety of cancers including breast (190), lung (191), colon (192), and ovarian cancers (193) as well as in Chronic Myelogenous Leukemia and Acute Myelogenous Leukemia (194, 195), respectively [for a full review, see (196)]. Similarly, STAT3 inhibition can enhance the efficacy of established chemotherapeutic agents and regimens (197) or in cancers that possess disrupted TGFβ signaling (198). Many types of STAT3 inhibitors have been developed thus far, including small molecule (199-201) and peptide (202, 203) inhibitors. However, no inhibitors targeting STAT3 have progressed to clinical trials yet; rather, current chemotherapeutic agents have been explored and postclinically demonstrated to have anti-STAT3 mechanisms. Examples of this are Satraplatin, a tetravalent platinum derivative (197), and Silibinin, an Erk1/Erk2 kinase inhibitor (204). It must be emphasized, however, that STAT3

is a common downstream effector of many upstream receptor and soluble kinases; and therefore many targeted agents are specific for upstream kinases of STAT3, rather than STAT3 itself. Many anticancer agents have been developed to target the IL-6 Receptor (IL-6R), Epidermal Growth Factor Receptor (20), Src, the Jak kinases, Tumor Necrosis Factor α (TNFα), Platelet-derived Growth Factor Receptor (PDGFR), and several others. Furthermore, a large number of these agents have progressed to clinical trials (205-219) but not to combination trials. Many future antitumor regimens are also possible that have not yet been tested.

STAT3 and TGFβ: Published Mechanisms of Cross-Talk

Although both TGFβ and STAT3 have been implicated in breast cancer growth and progression, the mechanism of cross-talk between these two pathways has not been extensively characterized. Several studies have provided evidence suggesting TGFβ to be a factor that suppresses STAT3 tyrosine phosphorylation or Interleukin-6 (IL-6) signaling (220).

However, the role of cross-talk between TGFβ and IL-6 induced STAT3 tyrosine phosphorylation has not been well established in the cancer milieu. One study reported TGFβ stimulation of STAT3 tyrosine phosphorylation (221), while another has demonstrated Smad4-mediated inhibition of tyrosine phosphorylated STAT3 in pancreatic cancers (222). However, the mechanisms responsible for these effects and that reconcile these various findings are currently not well characterized.

Epithelial-To-Mesenchymal Transition (EMT) and Breast Cancers

The process of EMT has been studied in breast cancer cell lines (223) but remains elusive *in vivo*. Strikingly, one group has demonstrated a role for the immune system in regulation of EMT *in vivo*, promoting the development of breast cancer stem cells (224). Another study has demonstrated the effect of EGFR and STAT3 cross-talk and concluded that this signaling can induce EMT due to STAT3 Ser (727) upregulation of the E-Cadherin repressor molecule Twist

(225). Notably, EGFR/IL-6/STAT3 cross-talk drives EMT in ovarian carcinomas (226) and breast carcinoma lines (227) through IL-6 dependent N-Cadherin upregulation.

TGFβ Induction of EMT Involves Diverse Pathways and Mechanisms

Researchers described TGFβ induction of Epithelial Mesenchymal Transition (EMT) first in the developmental process, in which EMT is critical for switching apical-basolateral polarity to differentiated tissue consisting of epithelial and mesenchymal cells (228). Later it became apparent that TGFβ stimulation of EMT was a process subverted by cancer cells to mediate invasion and metastasis (57). During EMT epithelial cells lose their polarity and their ability to form cuboidal, tight colonies, and develop into fibroblastic nonpolar single cells that poorly associate into colonies (229). In addition to changes in colony formation, the actin cytoskeleton is rearranged from cortical actin into parallel rigid F-actin fibers, termed stress fibers (230). Subsequent studies have identified various signaling molecules and pathways as being essential for both the molecular changes and for the changes in the cellular cytoskeleton induced by EMT. An important mediator of actin rearrangement, RhoA, and its downstream kinase ROCK are critical for TGFβ stimulation of the EMT program (229). Intact Smad signaling is required for TGFβ initiation of EMT, as are Erk2 (231) and the Erk pathway (232), NF kappa B (233), the EGF Receptor (234), and Snail (235) activity. Additional molecules and pathways have been identified as critical for EMT-dependent organogenesis in other systems (236-240) but will not be discussed herein.

TGFβ is a well-established inducer of stress fiber formation in cells that undergo EMT (57, 231, 241). This entails the reorganization of cortical actin into filamentous, parallel-oriented actin fibers, termed stress fibers (232). Actin structure and cellular signaling are closely linked, as many signaling molecules reside at the sites of focal adhesions, where cells attach to a substratum (242). Examples include the soluble Src kinase, which is well known to be an

activator of STAT3, as well as Focal Adhesion Kinase (92, 243). Forces such as mechanical shear stresses can alter cellular signaling in the absence of other ligand stimulation (244). One of the key morphological characteristics of TGFβ-stimulated EMT is a switch from cuboidal epithelial colonies to mesenchymal, elongated spindle-shaped cells that do not readily form colonies. Therefore, such a dramatic switch—which is due to TGFβ treatment in some systems—must necessarily engage a dramatic reorganization of the actin cytoskeleton (245, 246).

The Cadherin Switch: Mediating Changes in Cell-Cell Adhesion and Motility/Invasion

As part of the switch from an epithelial to a mesenchymal cellular phenotype, cells downregulate the adhesion molecule E-Cadherin and upregulate N-Cadherin (228). This process is mediated by the E-Cadherin repressor proteins Snail and Slug (247) and the Inhibitor of Differentiation proteins Id1 and Id2 (248), but is still poorly understood. E-Cadherin expressors form tight homophilic bonds and therefore form tighter colonies, while cells expressing N-Cadherin poorly associate into colonies and are more motile (249). In development the so-called "Cadherin Switch" is sufficient to induce segregation of cells expressing these molecules, but in cancers this event is not well understood. Several studies in nontransformed mouse epithelial cells have demonstrated that N-Cadherin expression, regardless of E-Cadherin expression levels, is sufficient for cellular invasion (250, 251).

Regulation of E- and N-Cadherin Expression: A Potential Role for STAT3

The process of Epithelial to Mesenchymal Transition (EMT) is one that is well characterized in terms of the "Cadherin Switch," or the downregulation of the epithelial adhesion molecule E-Cadherin, with concurrent upregulation of the mesenchymal adhesive N-Cadherin protein (249). These homophilic adhesion molecules, when expressed on their respective cell types, exhibit differential adhesiveness, with E-Cadherin junctions facilitating tight colony

formation (252). Conversely, mesenchymal cells expressing N-Cadherin do not readily form colonies (253), and many studies have been performed to elucidate the exact role of these two cadherins in migration and invasiveness *in vitro* (251, 254-256). For example, the presence of N-Cadherin appears to drive adhesive behavior, regardless of E-Cadherin expression status (250), at least in certain cell lines.

It is important to note that a major system for studying EMT has until recently been via TGFβ treatment of nontransformed mouse mammary NMuMG cells (58, 257). However, with the discovery that different signals (225, 226, 233, 235, 238, 258-261) can induce an EMT-like phenotype in multiple cell types (227, 236-238, 246, 262-270), scientists have gained more perspective on EMT.

Several studies have observed a correlation between STAT3 tyrosine phosphoryation and downregulation of E-Cadherin (165, 226, 227, 263, 271-273) and that activated STAT3 is also sufficient for upregulation of N-Cadherin (227, 272, 274). Further understanding of this mechanism is complicated by the fact that homotypic cell adhesions, and in particular E-Cadherin homodimeric complexes, can regulate STAT3 tyrosine phosphorylation (275). However, the mechanism responsible for STAT3-dependent upregulation of N-Cadherin has not been characterized in detail. In particular, the specific cytokines and growth factors responsible for these effects have not been implicated, nor has STAT3-mediated N-Cadherin upregulation been placed in the larger context of EMT or in the still more complex part EMT plays in carcinogenesis.

Rationale and Study Outline

Because of STAT3's varied roles in breast cancer promotion and progression, we hypothesized that treatment with TGFβ, which is an important component of the cancer milieu, would result in activation of the STAT3 pathway, thereby modeling a possible signaling scenario

in the carcinogenesis process. Thereafter, data suggesting this pathway occurs in invasive human breast cancers will emphasize the importance of TGFβ-STAT3 cross-talk. A major part of this study was to understand some of the cross-talk that occurs in breast cancers, particularly in the critical stages of its development, with the hope that future chemotherapeutic regimens and therapies may be developed based on our research.

With these objectives in mind, we designed our study with the purpose of characterizing the mechanism by which TGFβ induces STAT3 tyrosine phosphorylation. By examining the downstream characters responsible for TGFβ/Smad and IL-6/STAT3 signaling, we hypothesized that critical members of each pathway were required, and that rather than acting in a manner of cross-signaling, TGFβ was linearly inducing STAT3 tyrosine phosphorylation through the upregulation of Interleukin-6. Through examination of each of these interactions we sought to understand the relationship between TGFβ and STAT3.

Clinical Implications and Other Considerations

Due to the varied roles of TGFβ and STAT3 in carcinogenesis, particularly in the later stages of invasion and metastasis, many inhibitors of these two pathways have already been developed, and some are in various stages of clinical trials (93, 94, 212, 219). However, because cancer development occurs through overexpression and cross-activation of many different signaling pathways, it is more than probable that different signaling cross-talk occurs aberrantly. TGFβ and STAT3 cross-talk can occur in nontransformed mouse mammary epithelial cells as well as in a luminal human breast cancer cell line. Further, it is endogenous to a basal-like human breast cancer cell line, suggesting a role for TGFβ stimulation of STAT3 tyrosine phosphorylation in human breast cancer invasiveness.

The observation that TGFβ stimulates STAT3 tyrosine phosphorylation in human breast cancer cell lines has several clinical implications. Current inhibitors of TGFβ and the STAT3

pathway (including the IL-6 ligand and receptor, Jak, and STAT3 itself) may be used in combination therapy for enhanced efficacy compared to either treatment alone. Further, development of future agents targeting components of both these pathways—for example, a dual inhibitor prodrug that is hydrolyzed after entry into the cell, may thus target both the Jak and TGFβ kinase activity domains. Conversely, another strategy could be to inhibit extracellular components of this pathway, namely secreted TGFβ and IL-6. In these ways and many others, we hope that our signaling studies will provide a basis for development of highly optimized, efficacious anti-cancer therapeutics in the future.

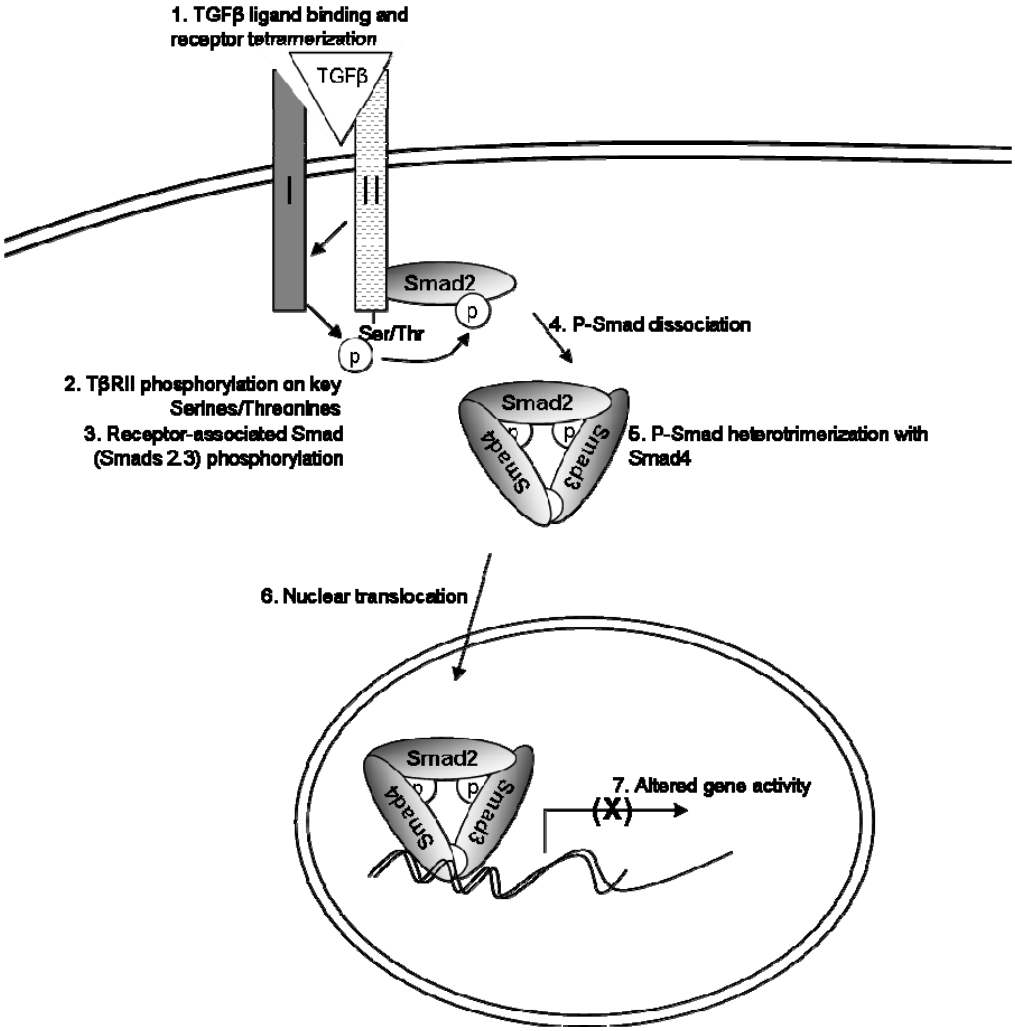

Figure 1-1. The TGFβ signaling pathway.

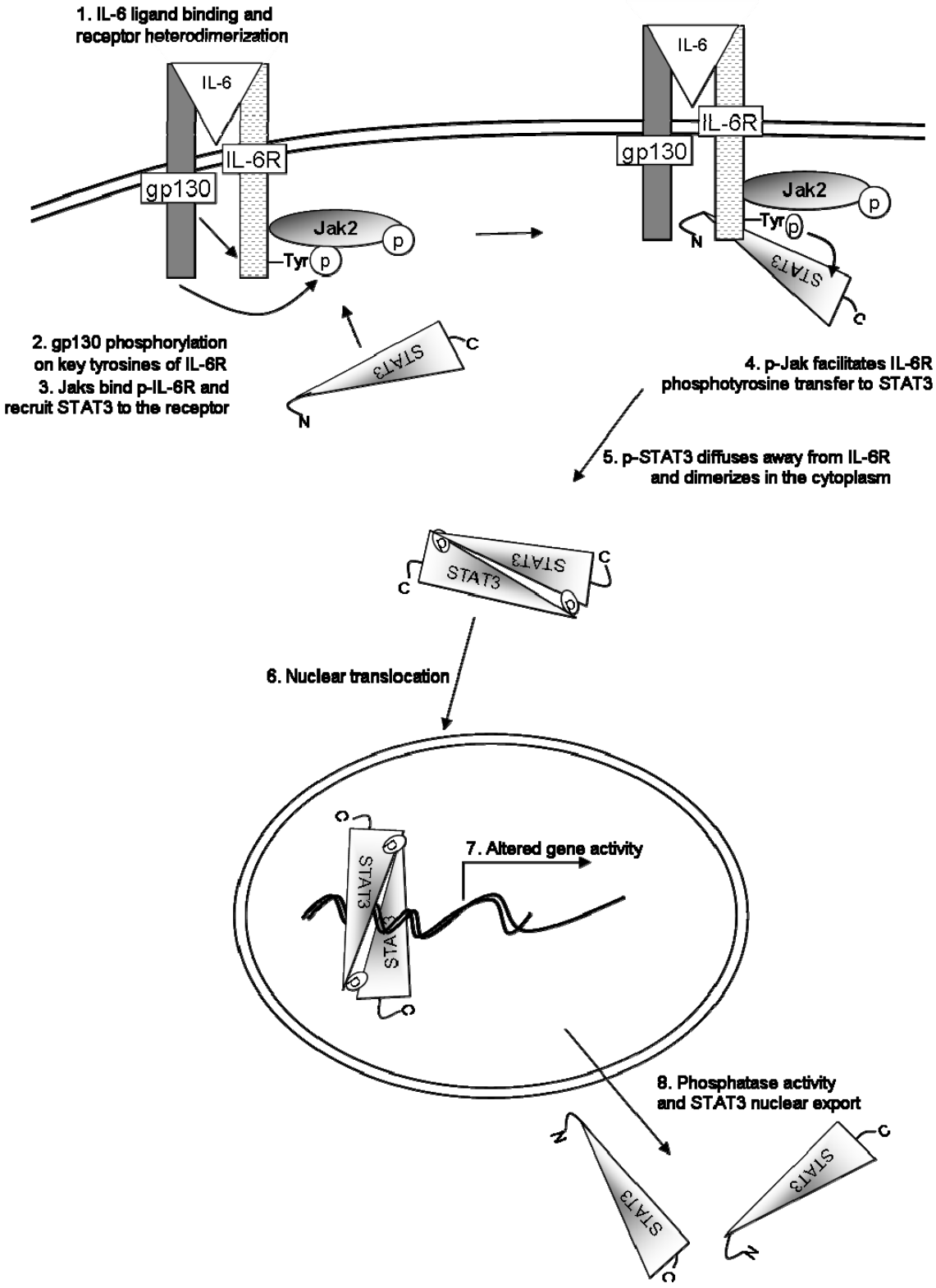

Figure 1-2. Cytokine stimulated STAT3 pathway.

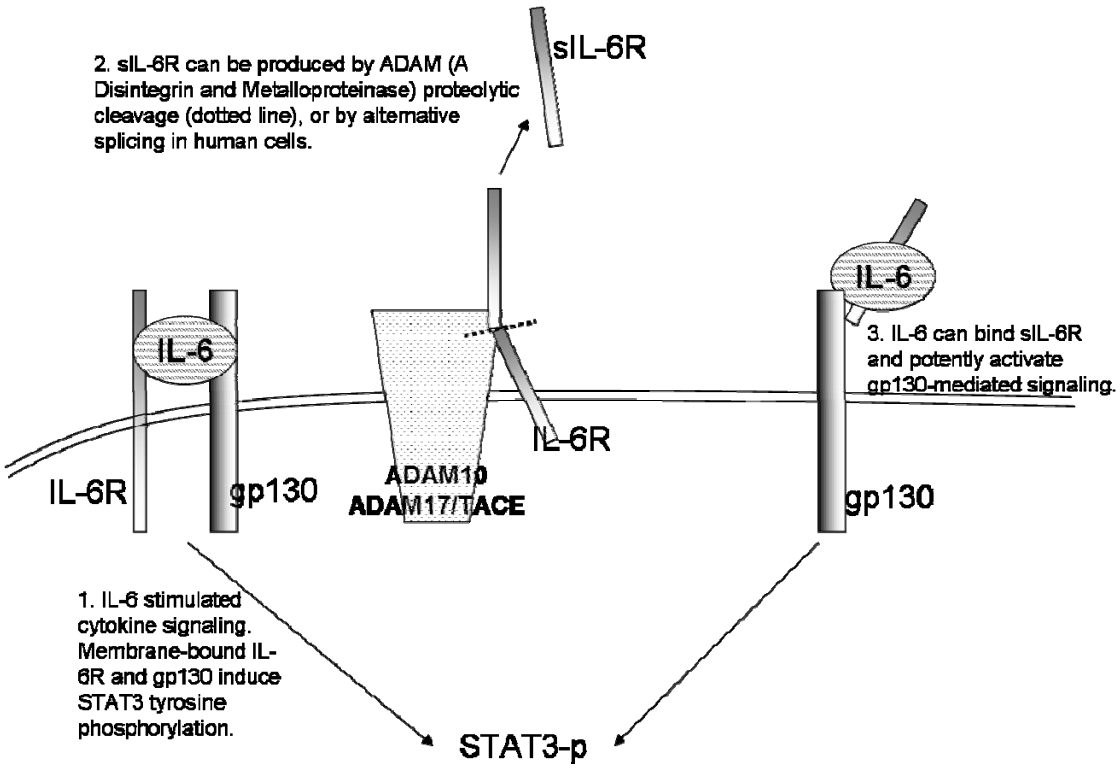

Figure 1-3. Mechanisms of IL-6 trans-signaling.

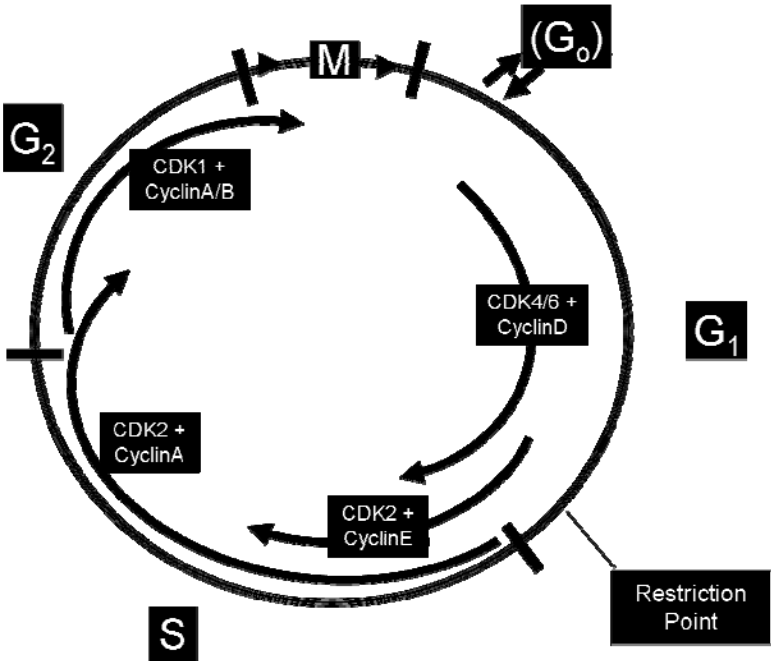

Figure 1-4. The classical cell cycle model.

CHAPTER 2
GENERAL MATERIALS AND METHODS

Tissue Culture, Maintenance, and Plating of Cell Lines

All cell lines were maintained in 10% FBS-DMEM at 37 ºC/5% CO_2. Cell cultures were maintained in the exponential growth phase. Cells were trypsinized and counted with a hemocytometer; for western blot experiments cells were typically plated at 350,000 cells per P_{100} plate and incubated overnight at 37 ºC. MMTV-D1K2 tumor-derived fibroblast lines (TDFs) were isolated as previously described (276).

Isolation of Mouse Mammary Tumor Associated Myofibroblast Cells and Maintenance of the TDF Cell Line

Mouse mammary tumors derived from a Cyclin D1-Cdk2 transgene (D1K2) driven by the Mouse Mammary Tumor Virus (MMTV) promoter were harvested as previously published (276-278). Tumor associated fibroblasts were collected by differential trypsinization. The epithelial tumor cell component was maintained as tumor lines, while the tumor-derived myofibroblast (TDF) cells were characterized as published (276).

Tritiated (^{3}H)-Thymidine Incorporation Assays

Cells were plated in 24-well plates. Treatments were for 24 h in triplicate. During the final two hours of treatment the cells were pulsed with ^{3}H-thymidine (Perkin Elmer, Boston, MA). Cells were fixed and washed twice with 10% trichloroacetic acid. After neutralization with 0.2 N sodium hydroxide, suspension aliquots were quantified for radioactivity in a Beckman Coulter scintillation counter. Data were normalized to controls.

Constructs, Drugs, and Reagents

TGFβ (Millipore, Billerica, MA), Epidermal Growth Factor (EGF, Chemicon, Billerica, MA), Leukemia Inhibitory Factor (LIF, Millipore), Vascular Endothelial Growth Factor (VEGF, Peprotech, Rocky Hill, NJ), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF,

Peprotech), basic Fibroblast Growth Factor (bFGF, Biosource, Camarillo, CA), Hepatocyte Growth Factor (HGF, Chemicon), Tumor Necrosis Factor α (TNFα, Chemicon), Leukemia Inhibitory Factor (LIF, Chemicon), Transforming Growth Factor α (TGF-α, Chemicon), Lysophosphatidic Acid (LPA, Sigma, St. Louis, MO), and sIL-6Ra (R&D Biosystems, Minneapolis, MN) were dissolved in PBS +1% BSA. Recombinant hIL-6 (Sigma) was dissolved in sterile water. The LPA receptor inhibitor VPC51299 was kindly provided by Dr. K. Lynch at the University of Virginia and was dissolved in PBS/DMSO. The inhibitors Stattic (Calbiochem, San Diego, CA), Jak Inhibitor I (Calbiochem), Jak2 inhibitor (AG490, Calbiochem), EGFR/Her2 Inhibitor (Calbiochem), TβRKI (Calbiochem), SIS3 (Sigma-Aldrich), and GM6001 (Calbiochem) were dissolved in DMSO. Apratoxin A (279-281) was kindly provided by Dr. H. Luesch (University of Florida College of Pharmacy, Gainesville, FL). Wild-type STAT3 construct was obtained from Addgene (Cambridge, MA) (courtesy of J. Darnell).

Inhibition of Actin Polymerization and of Various Signal Transduction Pathways

The actin depolymerizing agents Swinholide A, Cytochalasin D, Phalloidin, Latrunculin A, and Jasplakinolide (Calbiochem) were dissolved in DMSO according to the manufacturer's instructions. Blockade of the Akt pathway (LY29002, Calbiochem), the Rho kinase ROCK (Y27632, Calbiochem), Hsp90 (Geldanamycin, Calbiochem), and Casein Kinase (CK)1δ and CK1ε (IC261, Calbiochem) were for 24 h at 37 ºC. All inhibitors were dissolved according to the manufacturers' instructions.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Cells were plated as described in P_{100} culture dishes. After treatments cells were harvested in Trizol reagent (Invitrogen, Carlsbad, CA) and RNA was isolated according to the manufacturer's instructions. One microgram of RNA was reverse transcribed, and the resulting cDNAs were used for gene amplification by PCR with the following primers: Mouse β-Actin, as

in (254) (Forward 5'-GTGGGCCGCCCTAGGCACCAG-3', Reverse 5'-CTCTTTGATGTCACGCACGATTTC-3'); GAPDH (5'-GAAATGAGCTTGACAAAG-3', Reverse 5'- CTGGCATGGCCTTCCGTG-3'); mouse IL-6 (Forward 5'-ACCACTCCCAACAGACCTGT-3', Reverse 5'-TCCAGTTTGGTAGCATCCAT-3'); human IL-6 (Forward 5'-AGATTCCAAAGATGTAGCCG-3', Reverse 5'-TGCCTCTTTGCTGCTTTCAC-3'); mouse TNF α (Forward, 5'-ATCGGCTGGCACCACTAGTTG-3', Reverse 5'-CCAGACCCTCACACTCAGATCAT-3'); mouse/human IL-6R (Forward 5'-CTGCCCACATTCCTGGTWG-3', Reverse 5'-GCTGWTGTCATAAGGGCTC -3', where W is A/T); and human Cyclophilin, as in (282). PCR reaction products were generated as follows: melt 96 ºC 1 min, anneal 60 ºC 1 min, extend 72 ºC 1 min; β-Actin, as in (254); and mIL-6, melt 96 ºC 1 min, anneal 62 ºC 1 min, extend 72 ºC 15 sec. IL-6R PCR products were synthesized with the following touchdown PCR protocol: 95 ºC (30 sec), 73.5→ 63.5 ºC (45 sec, decreasing 0.5º per cycle), 72 ºC (1 min), 15 cycles; 95 ºC (30 sec), 54 ºC (45 sec), 72 ºC (1 min), 25 cycles. PCR products were visualized on a 2% agarose gel.

SDS-PAGE and Westen Blot Analysis

Cells were plated and treated, then extracts prepared as previously described (276). Proteins were resolved on sodium dodecylsulfate-polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblotting was performed with the following antibodies: Actin (Santa Cruz Biotechnologies, Santa Cruz, CA, 1:5000); STAT3 (Cell Signaling Technologies, Danvers, MA, 1:5000); pSTAT3-Y705 (Cell Signaling, 1:500); His_5 (Qiagen, Germantown, MD, 1:1000); Pim1 (Santa Cruz, 1:500); Src (sc-8995, 1:1000); pSrc-Y416 (Cell Signaling, #2101, 1:500); and pSrc-Y527 (Cell Signaling, #2105, 1:500). Goat anti-rabbit and anti-mouse IgG

secondary antibodies conjugated with alkaline phosphatase (Santa Cruz) allowed band visualization.

Conditioned Medium Experiments

NMuMG cells were plated as described and treated for 24 h with various treatments. After treatment the conditioned medium (CM) was collected and cleared by centrifugation of cells and debris. NMuMG CMs were stored at 4 ºC for subsequent assays. The R-1B cell line (34) lacks a functional TβRI and was treated with conditioned medium for examination of TGFβ-independent signaling (Figure 2-1).

Transient Transfections and Luciferase Reporter Assays

Cells were transfected with Lipofectamine (Invitrogen), GeneJuice (EMD Biosciences, Madison, WI), or NanoJuice (EMD Biosciences) according to the manufacturers' instructions. The STAT3-responsive m67-luciferase reporter construct (0.4 μg; courtesy of J. Darnell) (283), pSTAT-luciferase construct (Clontech, Mountain View, CA), or MMP9-luciferase construct (284) (690 bp, courtesy of D. Boyd, MD Anderson Cancer Center, TX) was transfected as indicated and treated for 24 h; 48 hours post-transfection, cells were lysed. Luciferase assays were performed in triplicate as previously described (285). Values were normalized to protein concentration.

Immunofluorescence Microscopy

Cells were plated on sterile glass coverslips at low density in 6-well plates. Treatments were for 24 h at 37 ºC. The next day the adherent cells were fixed with 1% paraformaldahyde followed by quenching with 50 mM ammonium chloride. All solutions were supplemented with 0.5% Triton X-100 detergent. Fixed cells were probed with primary antibodies (1:100) as for western blot analysis. Goat anti-mouse or rabbit secondary antibodies (1:200) conjugated with Cy3 and/or Fluorophore 488 (Vector Laboratories, Burlingame, CA), and DAPI staining (Vector

Laboratories), permitted concurrent visualization of nuclei and proteins. Images were captured on a Leica TCS SP2 camera (Wetzlar, Germany) using the Openlab software suite.

Functional Inhibition of IL-6 Function with the Receptor fusion protein mIL-6-RFP

Transient transfection of the construct mIL-6.Receptor Fusion Protein Inhibitor (mIL-6-RFP) (286), consisting of the ligand-binding extracellular domains of the IL-6R and gp130, was carried out with Lipofectamine (Invitrogen) according to the manufacturer's instructions. The human form of this construct has been characterized elsewhere (287, 288). Protein expression of the receptor fusion protein was monitored with immunoblot analysis with a His_5 antibody that binds the His_6 tag on the C-terminus of the fusion protein. Functional validation of the construct was demonstrated in the presence of IL-6, whose effect on STAT3 tyrosine phosphorylation was abrogated upon transfection and subsequent protein expression of the mouse IL-6 receptor fusion protein. Transient transfection of the receptor fusion protein was sufficient for validation studies, but a more permanent method of receptor fusion protein collection was required for subsequent assays.

Establishment of Clonal Cell Lines Stably Expressing the IL-6 Receptor fusion protein

293A cells were transfected with Lipofectamine as described above to stably express either pcDNA3 vector control or the IL-6 receptor fusion protein construct mIL-6-RFP. Positive clones were selected with 1.0 μg/ml G418 medium. Clones were isolated by serial dilution and screened for construct expression by His_5-antibody detection.

Antibody Array Analysis

Mouse Cytokine Array I (Ray Biotech, Norcross, GA) was purchased and performed according to the manufacturer's instructions (Figure 2-2). Briefly, NMuMG cells were plated at subconfluent levels in duplicate and incubated in the presence or absence of TGFβ for 24 or 48 h. After treatments, duplicate CMs and protein extracts were pooled. CM samples were diluted 1:1

in Ray Biotech Sample Diluent and placed on the array. Protein extracts were diluted as usual and immunoblotted with STAT3, pSTAT3, or Actin rabbit antibodies to confirm TGFβ-induced pSTAT3. Array slide images were obtained on a Typhoon 9410 laser scanner. Positive samples were bound to antibodies conjugated with a Cy3-equivalent signal, and this fluorescent signal was detected at 10 pixel resolution.

Boyden Chamber Cellular Invasion Assays

Invasion assay plates containing Matrigel-coated 8 μm-pore membrane well inserts (BD Biosciences, San Jose, CA) were fed with cells according to the manufacturer's instructions. An FBS gradient was set up across the membrane, with low (0.2% FBS-DMEM) or complete (10% FBS-DMEM) medium in the insert and bottom wells, respectively. All treatments were present in both chambers across the membranes. Cells and treatments were incubated for 24-72 h at 37 ºC; inserts were removed and the noninvaded cells removed with a cotton swab. The invaded cells were fixed with methanol and stained with crystal violet. Membranes were excised and mounted on microscope slides and invaded cells were counted.

Image Acquisition and Processing, Data Quantification, and Statistical Analyses

Images of agarose gels were captured with ImageQuant software. Immunofluorescent microscopy samples were photographed with a Leica TCS SP2 camera. Antibody array slides were scanned with a Typhoon 9410 laser scanner. All images were edited with the Adobe Photoshop software suite or densitometrically analyzed with the Image J program (NIH, Bethesda, MD). All numerical data were analyzed with the Microsoft Office Excel program. Statistical analyses were performed with GraphPad Prism software or Microsoft Excel. Statistics were calculated with the two-tailed Student's unpaired *t*-test.

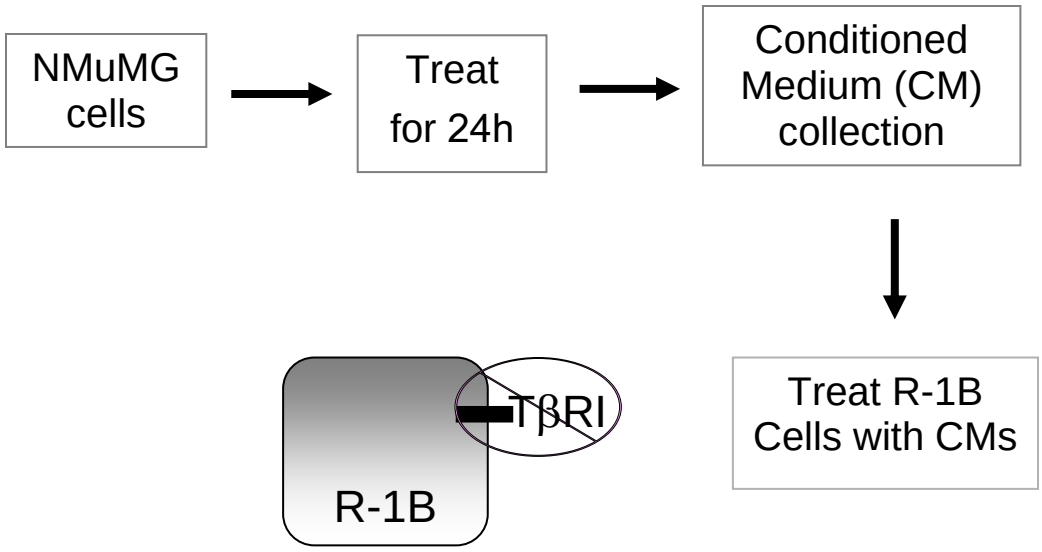

Figure 2-1. Design of Conditioned Medium (CM) experiments.

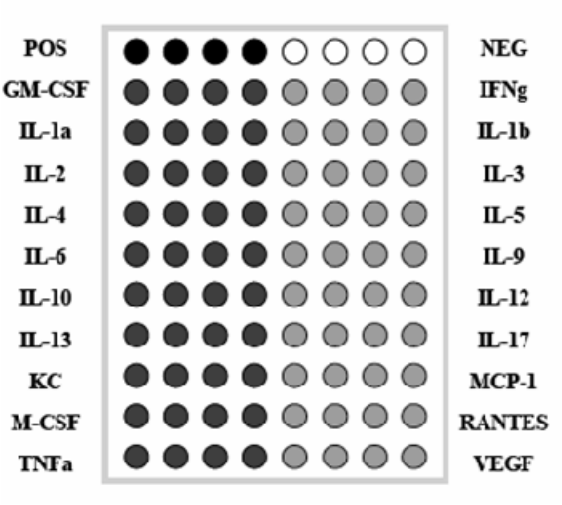

Figure 2-2. Mouse Cytokine I antibody array chip (Ray Biotech).

CHAPTER 3
TGF-BETA INDUCTION OF STAT3 TYROSINE PHOSPHORYLATION: MECHANISTIC DATA

Introduction

Published Cross-talk Between Members of the TGFβ and STAT3 Signaling Pathways

Previously, only a handful of studies have demonstrated a link between STAT3 and TGFβ signaling, and few of those studies have investigated this phenomenon in cancers. Several groups have demonstrated an inhibitory role of TGFβ on IL-6 signaling (220, 289), with particular emphasis on immune system modulation (290, 291) and development of the Th17 phenotype (292). One study focused on the effect of TGFβ on IL-6 expression in a prostate system (293), and another explored cross-signaling between TGFβ and IL-6 pathways in human trabecular cells (294).

TGFβ has varied effects, including immunosuppression, fibrosis, and wound healing (295-298). However, the role of TGFβ in carcinogenesis is still under debate due to its seemingly contradictory anti- and pro-tumorigenic functions (299-301). However, most data suggest a TGFβ Switch (302, 303) that signifies the point at which TGFβ functions are no longer tumor suppressive but oncogenic. TGFβ signaling is mediated primarily through the Smad proteins, which are phosphorylated on key serine/threonine sites (23).

Because STAT3 functions as an immunomodulatory factor, much of the research thus far has focused on this role. Quite separately, STAT3 has been heavily implicated in various stages and aspects of carcinogenesis (177, 187, 304) and is required for Src-mediated transformation (6, 7). STAT3 functions through phosphorylation of its activating Tyr (705) site (305). STAT3 phosphorylation induces formation of STAT3 homo- or heterodimerization with other STAT family members (101, 306), after which these dimers are translocated into the nucleus through importins and other proteins (138, 307). STAT3 dimers bind to STAT3-inducible elements

(SIEs), which are composed of the nuclear sequence AA(N_{4-5})TT, where N designates any base (308). Promoter-bound STAT3 dimers can cause either upregulation or downregulation of its target genes. Examples include upregulation of Bcl-2 (309), Myc and Survivin (118), p21 and Cyclin D1 (109), and downregulation of p53 (310) and Fas (311), which leads to enhanced growth rates, survival and evasion of apoptosis.

Lysophosphatidic Acid Signaling

Lysophosphatidic acid, or LPA, has been implicated as a negative prognostic marker in several types of cancer, including breast (312, 313) and ovary (312, 314). Elevated LPA levels in serum correlates with poor patient prognosis (315-317), while autotaxin/Phospholipase D, the catalytic enzyme responsible for LPA production from Phosphatidic Acid (PA), is also abundant in serum of cancer patients (316, 317).

LPA is a phospholipid that is produced from phosphatidic acid by the enzyme Autotaxin, also identified as Phospholipase D (PLD) (318). LPA binds its receptors, the LPA receptors LPA1-3, of the Edg family of receptors (319-321). Normal LPA signaling induces mitogenesis and differentiation through reorganization of the actin cytoskeleton due to Rho, Rac and Cdc42 activation (242, 322, 323), which are also stimulated by TGFβ (324, 325).

Considering the potential complexity of these pathways and potential downstream effects with regard to TGFβ and STAT3, we first examined the mechanism by which TGFβ induced STAT3 tyrosine phosphorylation. Initial experiments suggested that TGFβ treatment caused stimulation of STAT3 phosphorylation in an indirect manner, supported in large part by time course data. In line with this observation we hypothesized that a growth factor or cytokine was upregulated upon TGFβ treatment, and was itself responsible for STAT3 tyrosine phosphorylation. The studies in the following sections demonstrate this mechanism, as well as other characteristics that we observed in the course of these experiments.

Results

TGFβ Treatment Causes STAT3 Tyrosine Phosphorylation in Various Epithelial Cell Lines

Treatment with the growth factor TGFβ resulted in the phosphorylation of the activating Tyr (705) site of STAT3 (Figure 3-1). Significantly, this effect was observed in both nontransformed cell lines (e.g. NMuMG and Mv1Lu cells) as well as in various carcinoma lines (breast cancer lines MDA-MB-361, MMTV-D1K2-T1, and prostate cancer Du145 cells). We examined this mechanism primarily in nontransformed mouse mammary epithelial NMuMG cells, in which the effects of TGFβ have already been characterized in terms of Epithelial to Mesenchymal Transition (EMT) signaling (231). However, when R-1B cells (34), which lack a functional TGFβ Type I Receptor (TβRI), were treated with TGFβ no STAT3 tyrosine phosphorylation was observed, indicating that an intact TGFβ-TβRI signaling interaction is required (Fig. 3-2). This cell line was later used to examine TGFβ-independent signaling, as will be discussed in detail later on.

TGFβ stimulation of p-STAT3 occurred in a dose-dependent manner (Figure 3-2, left panel), with STAT3 tyrosine phosphorylation detectable at 0.1 ng/ml TGFβ treatment. In addition, treatment with 2.5 ng/ml TGFβ over time caused STAT3 phosphorylation after 4 h (Figure 3-2, right panel). Because direct growth factor signaling is usually completed within shorter time periods, we hypothesized that an indirect mechanism was responsible for TGFβ-induced STAT3 tyrosine phosphorylation.

TGFβ Stimulation of STAT3 Tyrosine Phosphorylation Does Not Occur Through Cell Cycle Arrest

Because TGFβ potently induces cell cycle arrest (326), we investigated whether cell cycle arrest was stimulating STAT3 phosphorylation. Cell cycle arrest with low serum (0.2% FBS-DMEM), TGFβ, or the microtubule inhibiting agent Nocodazole (500 nM) was quantified

by ^{3}H-thymidine incorporation and STAT3 tyrosine phosphoryation was monitored by immunoblot analysis. Although each of these treatments markedly arrested cells, only TGFβ treatment was sufficient to stimulate tyrosine phosphorylation of STAT3 (Figure 3-3). Therefore, we ruled out the possibility that cell cycle arrest was stimulating STAT3 tyrosine phosphorylation.

One hypothesis was that TGFβ signaling caused Src activation, as has been previously demonstrated (327). Therefore, we treated NMuMG cells with TGFβ and examined Src phosphorylation on Tyr (416) and Tyr (527), the activating and inhibitory Src phosphosites, respectively. We observed no significant increase in Tyr (416) phosphorylation, nor did we observe any decrease in Tyr (527) phosphorylation (Figure 3-4), indicating that Src does not significantly participate in TGFβ induction of STAT3 tyrosine phosphorylation.

TGFβ-Induced STAT3 Tyrosine Phosphorylation Requires Intact TGFβ, Jak, and Smad3 Signaling, But Does Not Require NF-κB Signaling

We hypothesized that TGFβ treatment was signaling through Smad3 and stimulating STAT3 tyrosine phosphorylation, and that STAT3 phosphorylation occurred through Jak kinase activity. We treated NMuMG cells with TGFβ in the presence or absence of a TGFβ Type I Receptor (TβRI) kinase inhibitor (TβRKI), a Jak inhibitor, or a specific Smad3 inhibitor (SIS3). Examination of STAT3 tyrosine phosphorylation by immunoblot revealed that inhibition of any of these molecules was sufficient to abrogate TGFβ stimulation of STAT3 tyrosine phosphorylation (Figure 3-5). Similarly, because TGFβ has been demonstrated to induce Nuclear Factor kappa B (NF-κB) signaling (293), we treated NMuMG cells with TGFβ in the presence or absence of three NF-κB inhibitors (Bay 11-7082, SN-50, or Parthenolide). Treatment with one or all of these inhibitors was not sufficient to inhibit TGFβ induction of

STAT3 tyrosine phosphorylation (Figure 3-6), suggesting that NF-κB signaling is not involved in this particular mechanism.

TGFβ Stimulation of STAT3 Tyrosine Phosphorylation Induces STAT3 Dependent Transcription

To examine downstream effects of TGFβ induction of STAT3 tyrosine phosphorylation, we transfected three STAT3-dependent luciferase constructs into NMuMG or HepG2 cells. The m67-luciferase construct (Courtesy of J. Darnell, (283)) was transfected into NMuMG cells, while the pSTAT (328) and MMP-9 (2.1 kb, courtesy of D. Boyd, MD Anderson Cancer Center (284)) luciferase constructs were transfected into HepG2 cells, which display a high transfection efficiency. Treatment with TGFβ was sufficient to induce STAT3 dependent luciferase activation approximately two-fold in each case (Figure 3-7) ($p=0.0109$, $p=0.011$, and $p=0.001$ for m67, pSTAT, and MMP9 luciferase activities, respectively, as measured by a two-tailed unpaired Student's *t*-test). These data suggested that TGFβ stimulation of STAT3 tyrosine phosphorylation induces STAT3-dependent transcriptional activity. Examination of STAT3-dependent genes was not performed because promoter sites of STAT3 target genes are also activated by other transcription factors, including the Smads, therefore complicating and potentially confounding upregulation data. However, these questions will be addressed in subsequent studies which lie beyond the scope of this particular project.

TGFβ-Induced STAT3 Tyrosine Phosphorylation Requires the Presence of Serum

To investigate whether growth factors in the serum could be responsible for TGFβ stimulation of STAT3 tyrosine phosphorylation, we treated NMuMG cells with TGFβ in the presence of either complete medium (10% FBS-DMEM) as in previous experiments, or with low serum (0.2% FBS-DMEM). As shown in Figure 3-8, serum is required for TGFβ stimulation of STAT3 tyrosine phosphorylation. Closer examination of samples treated with TGFβ in the

presence of low serum revealed marginal STAT3 phosphorylation, and complete medium alone was not sufficient to cause STAT3 tyrosine phosphorylation. We hypothesized that some factor present in serum was perhaps potentiating (or was potentiated by) TGFβ signaling. However, the maximal effect is achieved upon TGFβ and serum co-treatment, indicating that a threshold level of serum is required for significant levels of TGFβ induced STAT3 tyrosine phosphorylation.

Inhibition of Various Signaling Pathways Does Not Block TGFβ Stimulation of STAT3 Tyrosine Phosphorylation; Only Actin Depolymerization with Swinholide A Abrogates TGFβ-Induced STAT3 Tyrosine Phosphorylation

Because serum was required for TGFβ induction of STAT3 tyrosine phosphorylation, we hypothesized that there was a second participating pathway, perhaps induced by some serum factor, that together with TGFβ results in downstream STAT3 tyrosine phosphorylation. To examine this possibility, we screened a panel of growth factors for their ability to recapitulate STAT3 phosphorylation in the presence of TGFβ and low serum. As shown in Figure 3-9, it appeared that basic Fibroblast Growth Factor (bFGF), but not the other growth factors, was able to induce STAT3 phosphorylation in the presence of TGFβ and low serum. However, subsequent experiments did not support this finding, and we therefore ceased pursuit of this hypothesis.

Following the latter observation, we screened a panel of inhibitors for their ability to inhibit TGFβ stimulation of STAT3 tyrosine phosphorylation in the presence of serum. Our rationale was that if an inhibitor of Pathway X abrogated TGFβ-stimulated STAT3 tyrosine phosphorylation, then Pathway X was involved. Therefore, as shown in Figure 3-10, we inhibited the following proteins and pathways: STAT3, Src, ROCK, Actin polymerization, Hsp90, and Casein Kinases delta and epsilon. The STAT3 inhibitor Apratoxin A (279-281) and various actin depolymerizing agents (Jasplakinolide, Cytochalasin D, Latrunculin A, Swinholide A, and Phalloidin) were sufficient to inhibit TGFβ-induced STAT3 tyrosine phosphorylation.

We repeated the assay with the actin depolymerizing agents and found that only Swinholide A was sufficient to block STAT3 phosphorylation (Figure 3-11). The mechanism of action of this molecule involves depolymerization of actin filaments and sequestration of actin monomers in a 1:2 stoichiometric ratio (one molecule Swinholide A per two actin monomers) (329, 330). This different mechanism of action may explain why Swinholide A, but not other depolymerizing agents that work through different mechanisms, inhibits TGFβ stimulation of STAT3 tyrosine phosphorylation. Further examination of other signaling pathways will clarify whether Swinholide A is affecting signaling on a global level, or if there is some level of specificity for this agent. However, such experiments lie beyond the scope of this study.

Lysophosphatidic Acid (LPA) Treatment Can Fulfill the Serum Requirement for TGFβ Stimulation of STAT3 Tyrosine Phosphorylation

We hypothesized that Lysophosphatidic Acid (LPA) might be the factor present in serum that is responsible for TGFβ induction of STAT3 tyrosine phosphorylation. LPA is abundant in fetal bovine serum used to supplement tissue culture growth media (315, 331). Therefore, we treated NMuMG cells with TGFβ and LPA in the presence of low serum (Figure 3-12). The results suggest that serum depletion can be overcome by LPA treatment for TGFβ costimulation of STAT3 tyrosine phosphorylation. Notably, these effects were also blocked with Swinholide A (Figure 3-13). Although modest STAT3 phosphorylation is present in low serum with TGFβ treatment, we hypothesize that a full or maximal effect is achieved only when serum or LPA is also present.

LPA Receptor Inhibitor VPC-51299 Inhibits Serum and TGFβ Co-Stimulation of STAT3 Phosphorylation, But Not TGFβ-Induced IL-6 Upregulation

Thanks to the generosity of Dr. Kevin Lynch (University of Virginia), we pre-treated NMuMG cells with the LPA antagonist VPC51299 for 1 h and subsequently treated the cells with TGFβ and complete medium (10% FBS-DMEM) for 24 h. We expected inhibition of LPA

receptors and, therefore, abrogation of TGFβ induction of STAT3 phosphorylation. Indeed, LPA inhibition by VPC51299 pretreatment appeared sufficient to prevent or inhibit TGFβ and serum-induced STAT3 tyrosine phosphorylation (Figure 3-14). Future experiments, however, will examine the mechanism by which LPA signaling is responsible for TGFβ co-stimulation of STAT3 tyrosine phosphorylation. Because LPA induced signaling is complex and has varied downstream effects, a complex series of genetic and pharmacologic experiments will be required to implicate which LPA receptor is responsible, and which substrate downstream of that receptor(s) mediates STAT3 tyrosine phosphorylation. These experiments lie beyond the scope of this study.

Conditioned Medium from TGFβ-Treated NMuMG Cells Induces STAT3 Tyrosine Phosphorylation in the TβR1-Inactive R-1B Cell Line

We hypothesized from time course data that TGFβ treatment was inducing upregulation of some gene that was itself responsible for STAT3 tyrosine phosphorylation. To confirm this hypothesis, we performed Conditioned Medium (CM) studies in the R-1B cell line, which lacks functional TGFβ Type I Receptors (TβRI). We treated NMuMG cells with TGFβ or complete medium, collected the CM and treated R-1B monolayers with these CMs. Immunoblot analysis of the R-1B extracts suggested that NMuMG cells treated with TGFβ secrete some factor into the medium. Therefore, CM stimulation of R-1B cells allowed unknown factor-induced TβRI-independent STAT3 tyrosine phosphorylation (Figure 3-15). NMuMG-TGFβ-CM caused STAT3 tyrosine phosphorylation in R-1B cells whether R-1Bs were treated for 24 h (Figure 3-15, left panel) or 1 h (Figure 3-15, right panel) with the CMs. Therefore, TGFβ induction of STAT3 tyrosine phosphorylation involves some secreted factor that mediates downstream TβRI-independent STAT3 phosphorylation.

Leukemia Inhibitory Factor (LIF) Does Not Induce STAT3 Tyrosine Phosphorylation

The IL-6 family cytokine Leukemia Inhibitory Factor (LIF) has a significant role in mammary gland development, lactation, and post-lactational involution (332, 333). LIF signals through the interaction of the LIF receptor (LIFR) with gp130, which transduces signals to downstream effectors (102, 334, 335). Because LIF is secreted by breast cancer cells (189, 336-338), we hypothesized that TGFβ treatment could be inducing expression of this factor in NMuMG cells. We treated NMuMG cells with exogenous LIF and observed no STAT3 tyrosine phosphorylation, suggesting that the LIFR is not present at any appreciable levels in this cell line (Figure 3-16). Therefore, we ruled out the possibility that TGFβ stimulation of STAT3 tyrosine phosphorylation acts through secretion of the cytokine LIF.

TGFβ Induction of STAT3 Tyrosine Phosphorylation Is Mediated by IL-6 Secretion

We next treated NMuMG cells with two potential cytokine candidates, Interleukin-6 (IL-6) and Tumor Necrosis Factor α (TNFα). Exogenous IL-6 or TNFα treatment resulted in STAT3 tyrosine phosphorylation after 24 h (Figure 3-17). However, upon examination of IL-6 gene upregulation by RT-PCR, we observed that TGFβ treatment was sufficient to cause upregulation of IL-6, but not TNFα (Figure 3-18). TGFβ treatment was also sufficient to induce IL-6 upregulation in a time-dependent manner (Figure 3-19), with initial IL-6 upregulation detectable after 4 h of TGFβ treatment and increasing thereafter. Significantly, this pattern recapitulated the time-course of STAT3 tyrosine phosphorylation induced by TGFβ treatment.

IL-6 Treatment is Not Sufficient to Induce Morphological Changes Associated with EMT

Although IL-6 and TGFβ both induce phosphorylation of STAT3 on Tyr (705), only TGFβ has been demonstrated to induce Epithelial to Mesenchymal Transition (EMT) in NMuMG cells *in vitro* (326). Therefore, we treated these cells with TGFβ or IL-6 and photographed the cells over subsequent days. We observed that TGFβ-treated NMuMG cells undergo overt

morphological changes associated with EMT, specifically a change from cuboidal epithelial cells forming well-delineated colonies to spindle-shaped mesenchymal-like cells that do not form colonies. Significantly, IL-6 treatment did not reproduce this effect, even after four days of treatment. Instead we observed that IL-6 treated NMuMG cells formed radially-arranged colonies rather than the classical cobblestone appearance displayed by epithelial cells (Figure 3-20). We concluded that IL-6 treatment, even at longer time points, does not induce morphological cellular changes associated with EMT. This conclusion was significant in our choice of cell line for subsequent invasion assays. To distinguish between cells that have undergone EMT (which are generally more motile (57)), and those with TGFβ-stimulated STAT3 tyrosine phosphorylation, we used the Mv1Lu cell line in addition to NMuMG cells for invasion assays. These results are discussed in detail later.

Functional Inhibition of IL-6 with the Receptor fusion protein mIL-6-RFP Abolishes TGFβ-Induced STAT3 Phosphorylation

Because data thus far indicated that TGFβ treatment induces STAT3 tyrosine phosphorylation and that TGFβ treatment causes IL-6 upregulation (Figure 3-21), we hypothesized that inhibition of IL-6 would abrogate TGFβ-induced STAT3 phosphorylation. We obtained a mouse IL-6 receptor fusion protein (courtesy of Dr. G. Müller-Newen, University of Aachen) consisting of the extracellular domains of gp130 and the IL-6 receptors (IL-6R), and which has been characterized elsewhere (286-288). After transient transfection of the mouse IL-6 Receptor Fusion Protein (mIL-6-RFP) plasmid into 293A cells, the receptor fusion protein was expressed at the protein level and was detectable by immunoblot due to the C-terminal His_6 tag (Figure 3-22). Further, expression of this protein was sufficient to inhibit IL-6 stimulated STAT3 tyrosine phosphorylation (Figure 3-19, right panel). We constructed a cell line stably expressing the mIL-6-RFP fusion protein, which is approximately 100 kilodaltons (Fig. 3-23).

Further, we isolated clonal cell lines from the parental polyclonal population. Clone 7 of the 293A/mIL-6-RFP cell lines expressed the highest level of the His-tagged receptor fusion protein protein compared to the multiclonal (MC) population (Figure 3-24), and this clonal line was maintained and used for all IL-6 inhibition experiments thereafter.

Conditioned Medium (CM) from the 293A/mIL-6-RFP-CL7 cell line was collected and used to treat NMuMG cells in the presence or absence of TGFβ. CM containing mIL-6-RFP was sufficient to abrogate TGFβ-stimulated STAT3 phosphorylation in NMuMG cells (Figure 3-25). Taken together, inhibition of IL-6 attenuated STAT3 phosphorylation, suggesting that TGFβ stimulation of IL-6 causes downstream STAT3 phosphorylation.

TGFβ Treatment Confers an IL-6 Dependent Invasive Phenotype on the Nontransformed NMuMG and Mv1Lu Cell Lines

Mv1Lu and NMuMG cells were not inherently invasive as measured by cellular invasion through a Matrigel matrix coated 8 μm-pore membrane; however, treatment with TGFβ significantly (Control versus TGFβ samples: NMuMG cells, $p=0.0295$; Mv1Lu cells, $p=0.0023$, as measured by unpaired Student's *t*-test) increased cell invasiveness, which was abrogated with the IL-6 receptor fusion protein in both NMuMG (Figure 3-26) and Mv1Lu (Figure 3-27) cell lines. Similarly, treatment with IL-6 resulted in increased invasiveness (Control versus IL-6 treatment, $p=0.0043$, Student's *t*-test) that was abrogated by the IL-6 receptor fusion protein (Figure 3-28), indicating that IL-6 is necessary and sufficient for NMuMG invasion *in vitro*.

TGFβ Treatment Induces IL-6 Dependent Upregulation of the Cell Adhesion Molecule N-Cadherin

Treatment of NMuMG cells with TGFβ induces activation of Epithelial to Mesenchymal Transition (EMT). This program includes changes in morphology and in molecular marker expression, such as the adhesion molecules E-Cadherin and N-Cadherin (228, 249). Upon treatment with TGFβ in the presence or absence of the IL-6 receptor fusion protein mIL-6-RFP,

we observed an increase in N-Cadherin expression that was dependent on IL-6 signaling (Figure 3-29). N-Cadherin expression increased with IL-6 treatment, indicating that IL-6 is sufficient for N-Cadherin expression. Further, IL-6 inhibition was sufficient to inhibit basal and TGFβ-induced N-Cadherin expression. No major changes in E-Cadherin expression were observed with TGFβ treatment and IL-6 blockade; however, because all treatments were for 24 h, it is possible that greater differences might be observed with longer treatment times and therefore a EMT program that is further progressed.

IL-6 Dependent N-Cadherin Transcription is Not Necessary or Sufficient for EMT

Although IL-6 inhibition is sufficient to block TGFβ-induced upregulation of N-Cadherin, examination of the cellular morphology of NMuMG cells in the presence of different treatments indicated that IL-6 inhibition, and subsequently abrogation of N-Cadherin upregulation, was not sufficient to inhibit the morphological changes associated with EMT (Figure 3-30). Further, although N-Cadherin is a major factor in the EMT process (251, 339), overt blockade of N-Cadherin expression by IL-6 inhibition does not block EMT, indicating that other markers and processes are responsible for the major morphological changes induced by TGFβ in NMuMG cells. In addition, examination of the subcellular localization of N- and E-Cadherin by immunofluorescence microscopy confirmed that N-Cadherin upregulation is IL-6 dependent, as IL-6 treatment is sufficient to induce expression of this protein (Figure 3-31). TGFβ treatment induced upregulation of N-Cadherin, concomitant with internalization of E-Cadherin into the cytoplasm. Taken together, these studies indicated that TGFβ induction of N-Cadherin expression is IL-6 dependent, and that inhibition of N-Cadherin expression is dispensable for the progression of morphological changes associated with EMT.

TGFβ Induces VEGF and GM-CSF Secretion

We demonstrated earlier that TGFβ treatment induces STAT3 tyrosine phosphorylation in NMuMG cells, and that this phosphorylation is likely due to the secretion of IL-6. To quantify IL-6 secretion we performed antibody analysis of a mouse cytokine array chip from Ray Biotech. We observed that VEGF and GM-CSF were secreted in a time-dependent manner after TGFβ treatment, both cytokines accumulating over 48 h (Figure 3-32). However, no IL-6 secretion was detected. This negative result may be due to assay error, although a mechanism driven by a homologous cytokine or factor effect was not ruled out by this finding. We therefore pursued other methods of implicating IL-6 protein upon TGFβ treatment, and did not rely on ELISA/antibody-array type technologies.

To confirm that VEGF and GM-CSF were not the cytokines responsible for TGFβ stimulation of STAT3 tyrosine phosphorylation, we treated NMuMG cells with exogenous VEGF or GM-CSF to determine whether NMuMG cells can respond to these cytokines. There was no indication that the NMuMG cells responded to these cytokines in terms of STAT3 tyrosine phosphorylation (Figure 3-33), therefore discounting the involvement of these two cytokines in our proposed mechanism.

These data caused us to consider an alternative hypothesis: the secreted factor wasn't IL-6 but the soluble form of the IL-6R (sIL-6R), or the truncated extracellular IL-6R domain. This would account for TGFβ stimulation of STAT3 tyrosine phosphorylation through ligand-independent IL-6R trans-signaling, and this might account for the absence of IL-6 cytokine present in the antibody array results. Moreover, if IL-6R could oligomerize with the mIL-6-RFP fusion protein, its activity or function might be blocked and therefore abrogate TGFβ induction of STAT3 tyrosine phosphorylation.

Matrix Metalloproteinase Inhibition Does Not Block TGFβ Stimulation of STAT3 Tyrosine Phosphorylation

Because we hypothesized that TGFβ stimulation of STAT3 tyrosine phosphorylation was acting through expression and cleavage to generate the soluble form of IL-6R, we opted to block the activity of ADAM-family proteases by using a Matrix Metalloproteinase (MMP) inhibitor. The ADAM proteins proteolytically generate such active proteins as the IL-6R and TNFα (158, 159). Therefore, we treated cells with the pan-MMP small molecule inhibitor GM6001 (340). However, blockade with GM6001 did not abrogate TGFβ induction of STAT3 tyrosine phosphorylation (Figure 3-34), indicating that the MMP family ADAM proteins, or other MMP family members, were not contributing to this signaling event.

TGFβ Treatment Induces IL-6R Expression

As previously mentioned, one possible mechanism of STAT3 phosphorylation was that TGFβ treatment causes upregulation of the IL-6 receptor (IL-6R). Numerous studies have already suggested that the soluble form of the IL-6R (sIL-6R) displays agonist activity when bound to IL-6 (148); this mechanism could also be occurring in TGFβ-treated NMuMG cells if a basal level of IL-6 is also present from serum. Indeed, TGFβ treatment did result in upregulation of IL-6R as examined by RT-PCR (Figure 3-35). Because alternative splicing generates a soluble IL-6R form in human but not mouse cells, we were able to conclude that any resulting IL-6R protein is membrane-localized, and that any soluble IL-6R would be obtained only by post-translational modification, i.e., proteolysis. Notably, the IL-6R that we observed by RT-PCR was precursor mRNA, and is not yet specified as membrane-bound or soluble IL-6R protein. Therefore, we were unable to differentiate between these two localized forms at the RNA level.

Exogenous sIL-6Rα Does Not Recapitulate TGFβ Stimulation of STAT3 Tyrosine Phosphorylation

Treatment of NMuMG cells with recombinant mouse soluble IL-6 receptor (sIL-6Rα) did not fully recapitulate STAT3 tyrosine phosphorylation. Specifically, sIL-6Rα alone slightly increased STAT3 tyrosine phosphorylation (Figure 3-36, left panel), but TGFβ and sIL-6Rα cotreatment did not induce a synergistic effect on STAT3 tyrosine phosphorylation. Further, cotreatment of NMuMG cells with the receptor fusion protein and sIL-6Rα did not inhibit STAT3 tyrosine phosphorylation (Figure 3-36, right panel), suggesting that sIL-6Rα does not oligomerize with the receptor fusion protein; or, if it does, that it still retains signal transducing ability even in the presence of this fusion protein. Taken together, these results do not corroborate previous data, which led us to reconsider our hypothesis.

Based on these data we concluded that although TGFβ treatment causes upregulation of IL-6R at the gene transcription level, treatment with exogenous sIL-6R does not recapitulate the effects stimulated by TGFβ treatment. We therefore refocused our efforts on IL-6, and not IL-6R, in this mechanism.

Conclusions and Discussion

We have demonstrated in this chapter that the nontransformed NMuMG and Mv1Lu cell lines respond to TGFβ treatment by phosphorylation of STAT3 on its activating Tyrosine (705) site. The significance of this observation is twofold: not only is an indirect mechanism responsible for this phosphorylation event, but there is a significant downstream effect on STAT3-dependent transcription and cellular invasion. Stimulation with TGFβ results in upregulation of IL-6, which is the main cytokine inducer of STAT3 tyrosine phosphorylation in many systems. We have demonstrated that blockade of IL-6 function with a specific receptor fusion protein abrogates TGFβ induction of STAT3 phosphorylation. IL-6 is also sufficient to

induce cellular invasiveness in NMuMG cells. Further, interruption of the TGFβ-IL-6-STAT3 signaling loop inhibits the cellular invasion conferred by TGFβ treatment in NMuMG and Mv1Lu cells. Finally, TGFβ treatment causes N-Cadherin upregulation, which data suggest is IL-6 dependent. This upregulation may play a part in TGFβ (and therefore, in IL-6) dependent invasiveness.

The significance of these observations holds many implications for human cancers. We have observed TGFβ to induce STAT3 tyrosine phosphorylation after 4 h of TGFβ treatment. Significantly, longer exposures to TGFβ—as suggested by our data—may be a predisposing factor in the milieu of nontransformed cells *in vivo* to help promote neoplasia or a locally invasive and perhaps metastatic phenotype. That is, more chronic TGFβ exposures may be a contributing factor for neoplastic cells to acquire invasive properties.

One significant implication from these observations is the fact that the mechanisms contributing to a tumor cell's transition from hyperplastic to neoplastic states is currently not well characterized. We hypothesize that TGFβ may play a significant part in this progression, as TGFβ is known to contribute to both migration (257) and cellular invasiveness (76, 341), as we have observed in this study.

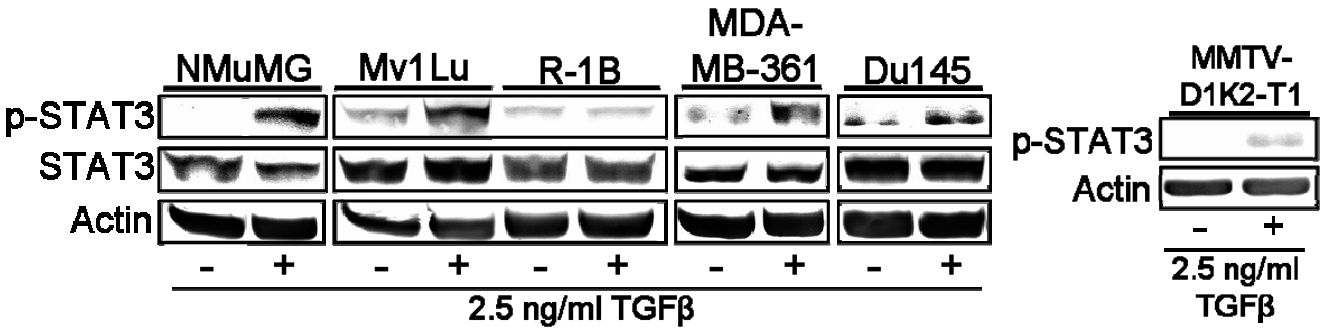

Figure 3-1. TGFβ treatment stimulates STAT3 Tyr (705) in various epithelial cell lines.

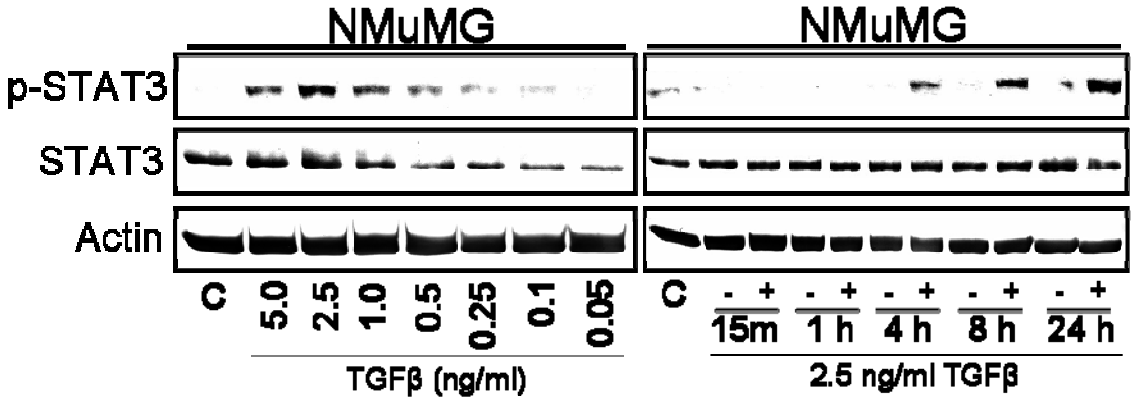

Figure 3-2. TGFβ-stimulated STAT3 tyrosine phosphorylation is dose- and time-dependent.

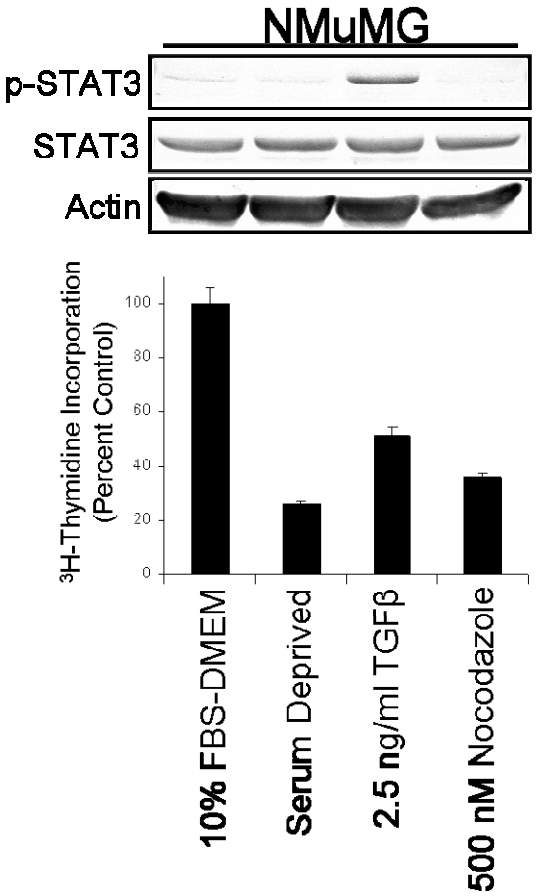

Figure 3-3. Cell cycle arrest is not sufficient for STAT3 tyrosine phosphorylation.

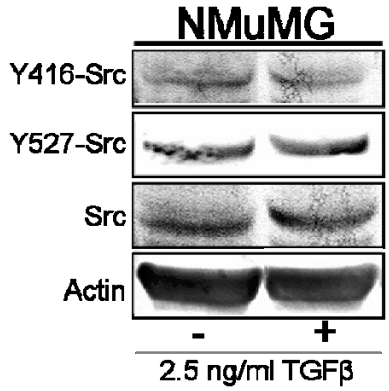

Figure 3-4. TGFβ treatment does not affect Src phosphorylation status in NMuMG cells.

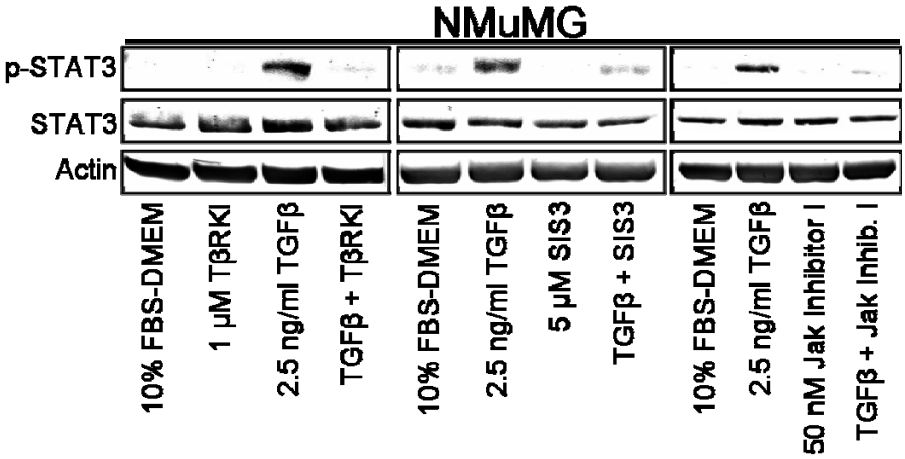

Figure 3-5. TGFβ stimulation of STAT3 tyrosine phosphorylation requires intact TGFβ/Smad3 and Jak signaling.

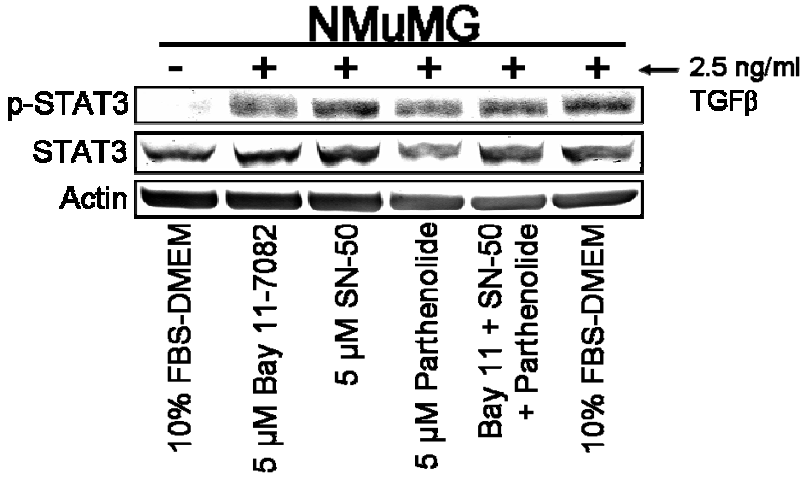

Figure 3-6. NF-kB activity is not required for TGFβ stimulation of STAT3 tyrosine phosphorylation.

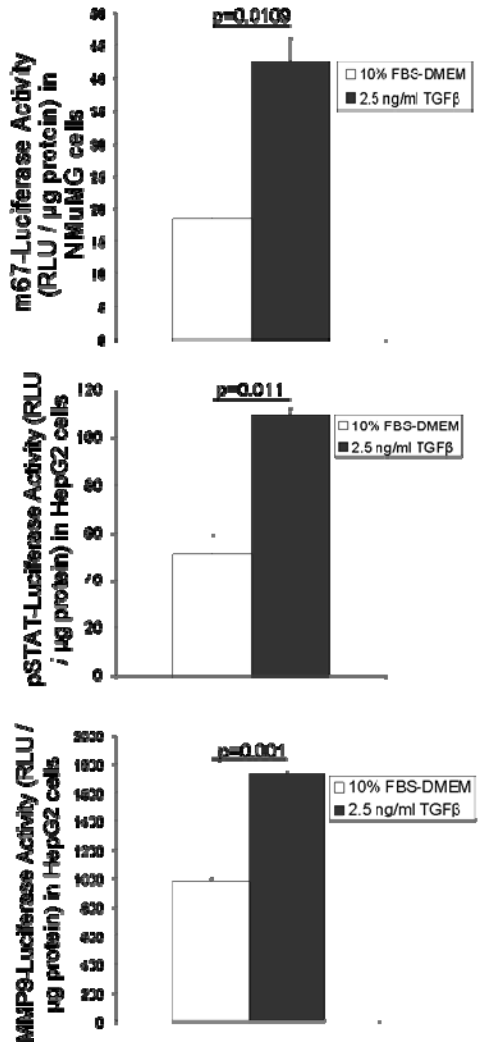

Figure 3-7. TGFβ activates STAT3 dependent gene transcription as measured by luciferase constructs.

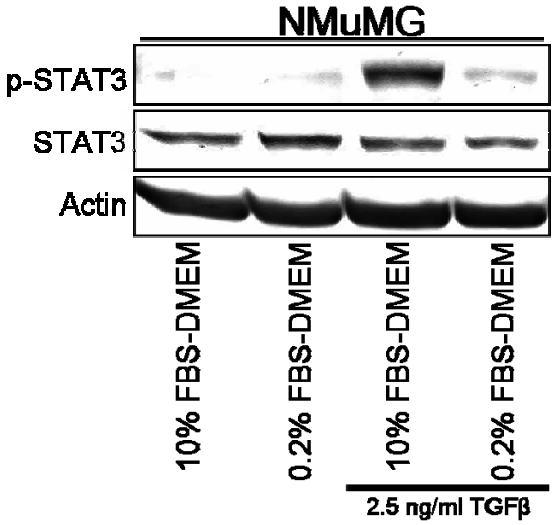

Figure 3-8. TGFβ stimulation of STAT3 tyrosine phosphorylation requires the presence of serum.

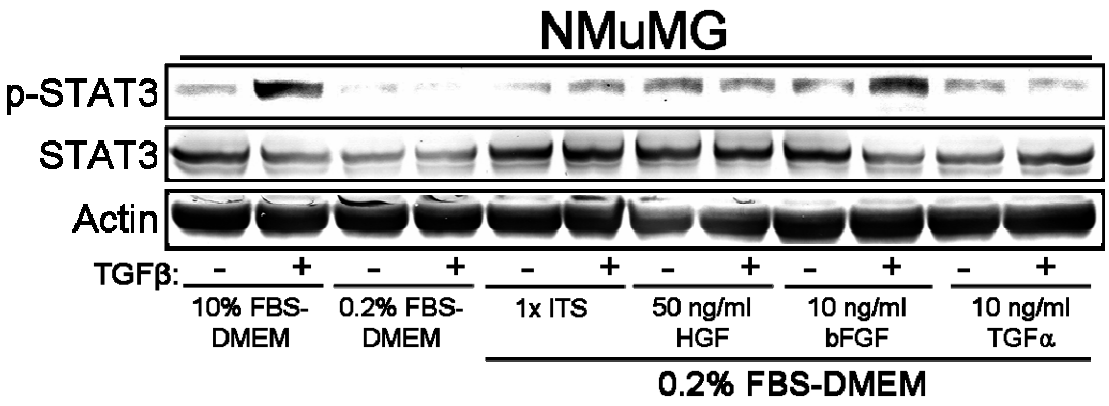

Figure 3-9. Reintroduction of various growth factors does not fulfill the serum requirement necessary for TGFβ induced STAT3 phosphorylation.

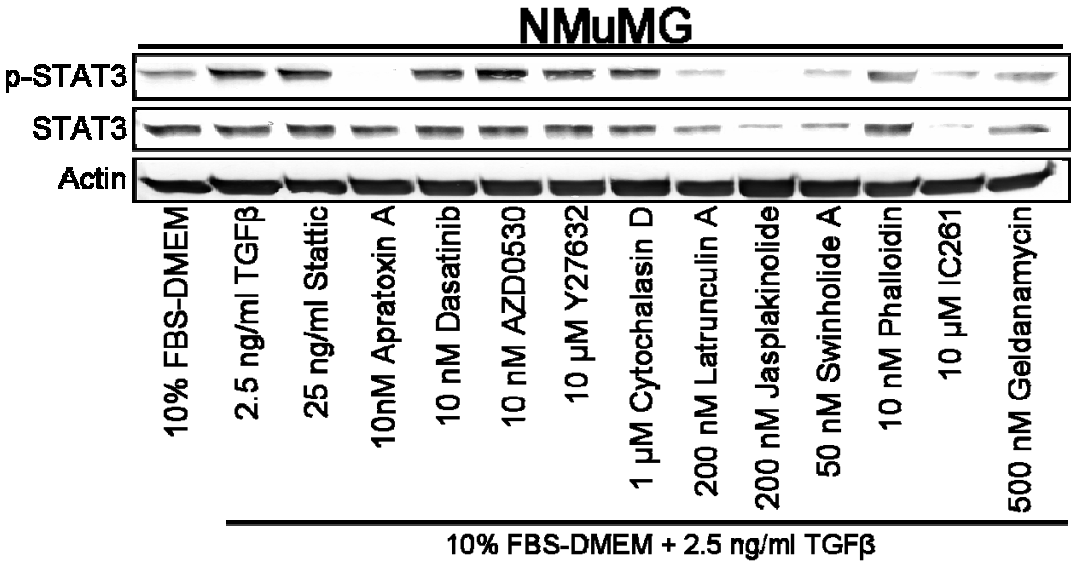

Figure 3-10. Inhibition of various signaling pathways does not block TGFβ-induced STAT3 tyrosine phosphorylation.

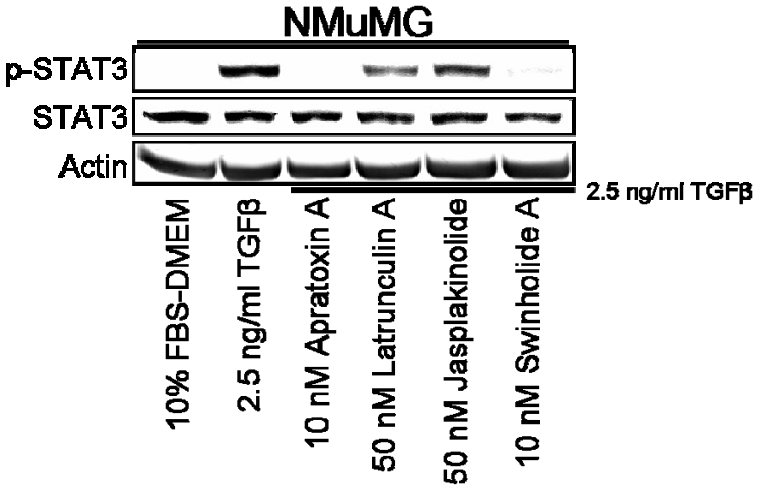

Figure 3-11. Swinholide A treatment, but not treatment with other actin depolymerizing agents, is sufficient to abrogate TGFβ induction of STAT3 tyrosine phosphorylation.

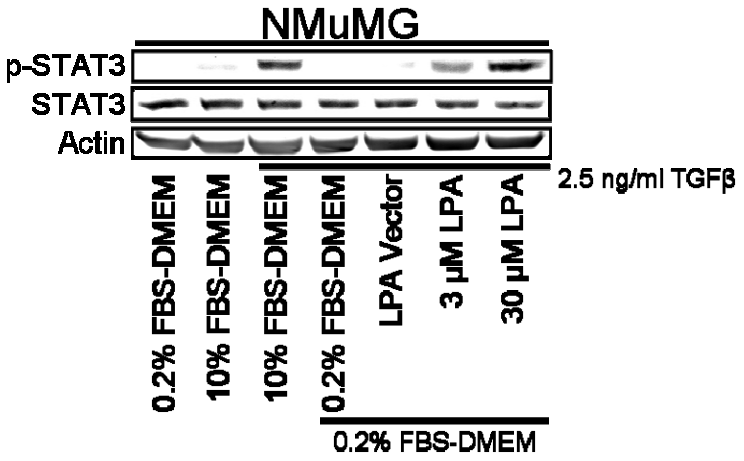

Figure 3-12. Lysophosphatidic Acid (LPA) can replace serum in TGFβ stimulation of STAT3 tyrosine phosphorylation.

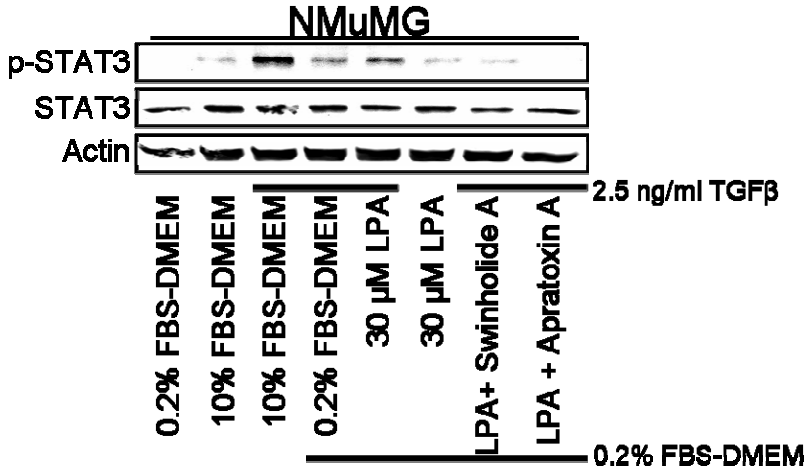

Figure 3-13. Treatment with Swinholide A is sufficient to inhibit LPA and TGFβ-induced STAT3 tyrosine phosphorylation.

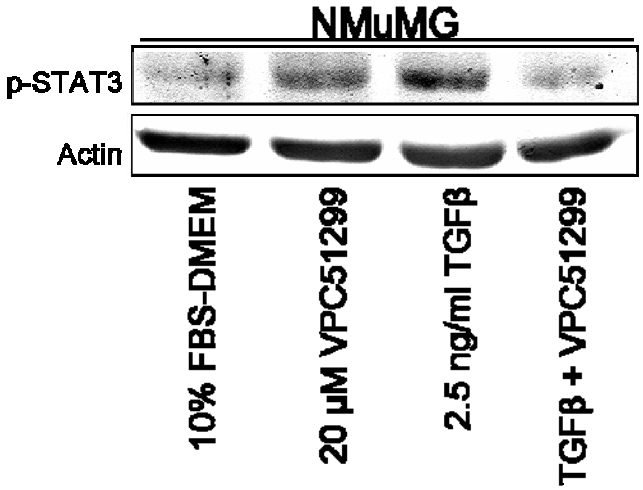

Figure 3-14. The LPA receptor antagonist VPC51299 blocks serum and TGFβ costimulation of STAT3 tyrosine phosphorylation.

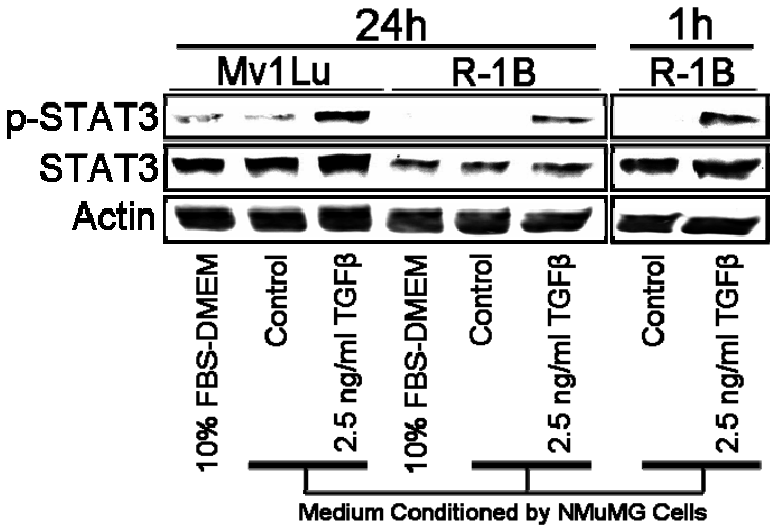

Figure 3-15. TGFβ treatment of NMuMG cells yields Conditioned Medium (CM) containing some factor capable of inducing STAT3 tyrosine phosphorylation in a TβRI-inactive cell line.

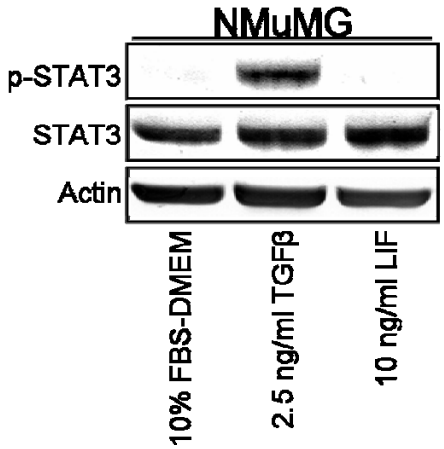

Figure 3-16. Leukemia Inhibitory Factor (LIF) does not induce STAT3 tyrosine phosphorylation in NMuMG cells.

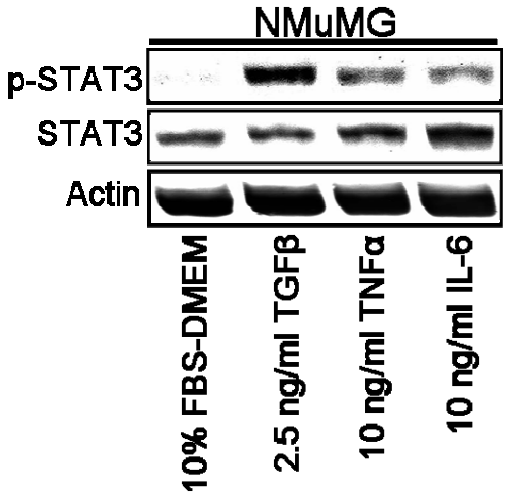

Figure 3-17. TNFα and IL-6 Both Induce STAT3 Tyrosine Phosphorylation in NMuMG Cells.

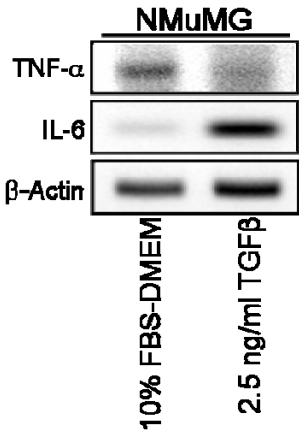

Figure 3-18. TGFβ induces Interleukin-6 (IL-6) upregulation.

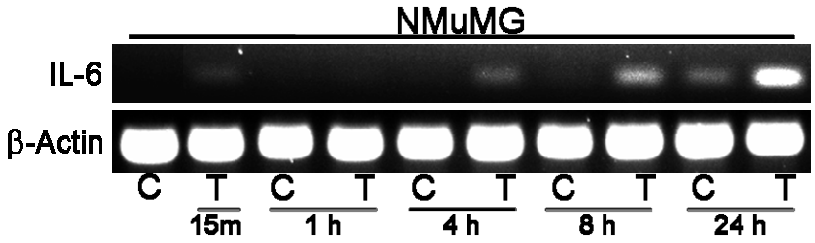

Figure 3-19. TGFβ induced Interleukin-6 (IL-6) upregulation is time-dependent.

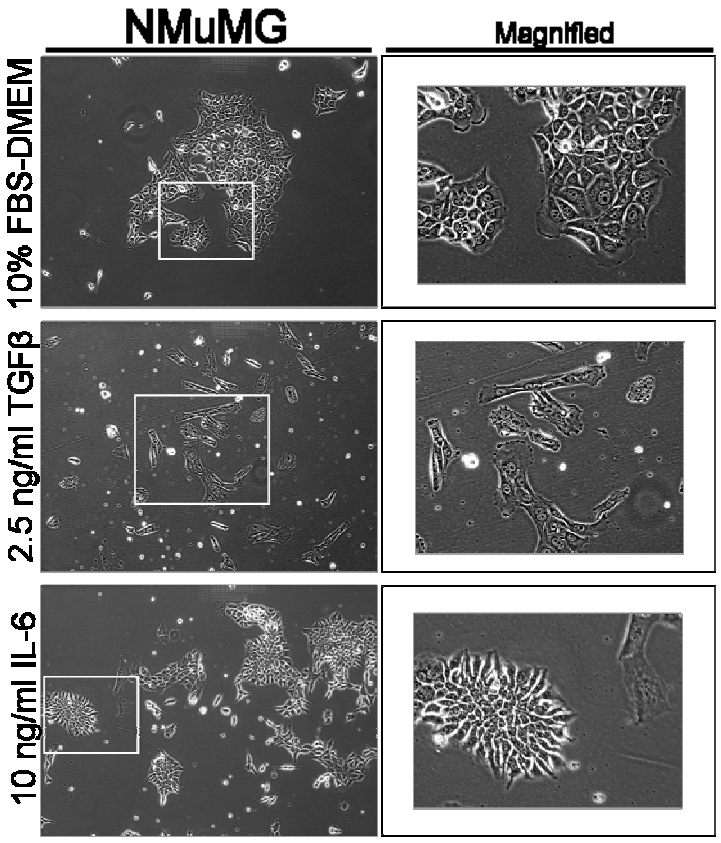

Figure 3-20. IL-6 does not recapitulate morphological changes associated with TGFβ-stimulated EMT.

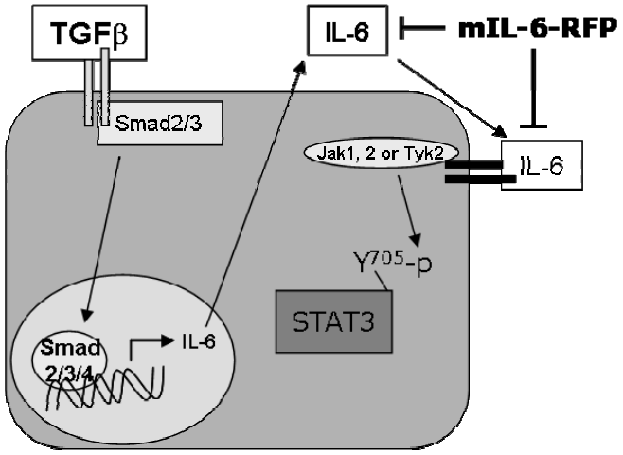

Figure 3-21. Tentative pathway describing TGFβ stimulation of STAT3 tyrosine phosphorylation.

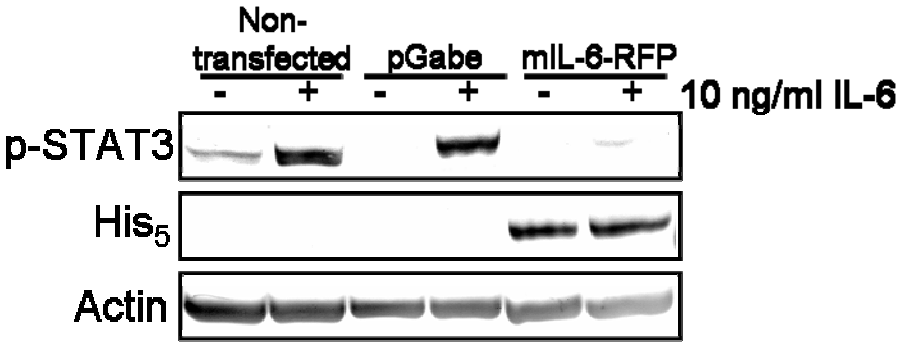

Figure 3-22. Characterization of the mouse IL-6 receptor fusion protein, mIL-6-RFP.

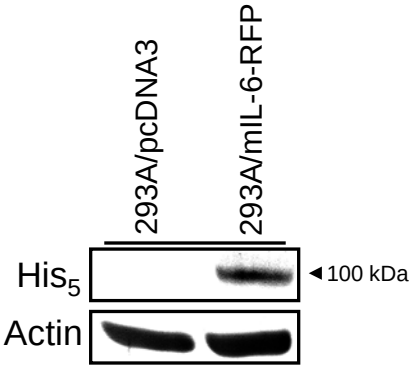

Figure 3-23. Establishment of 293A cell lines stably expressing the IL-6 receptor fusion protein.

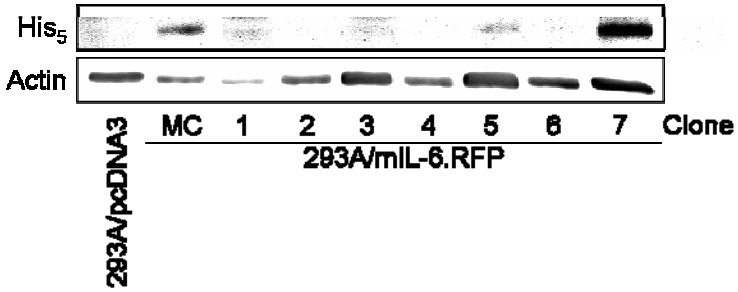

Figure 3-24. Isolation of clonal cell lines stably expressing the IL-6 receptor fusion protein.

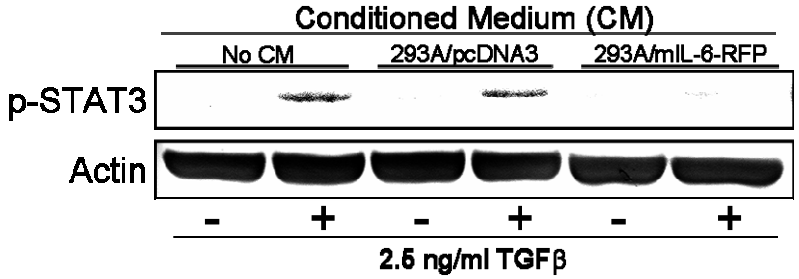

Figure 3-25. IL-6 functional inhibition by the receptor fusion protein mIL-6-RFP blocks TGFβ-induced STAT3 tyrosine phosphorylation.

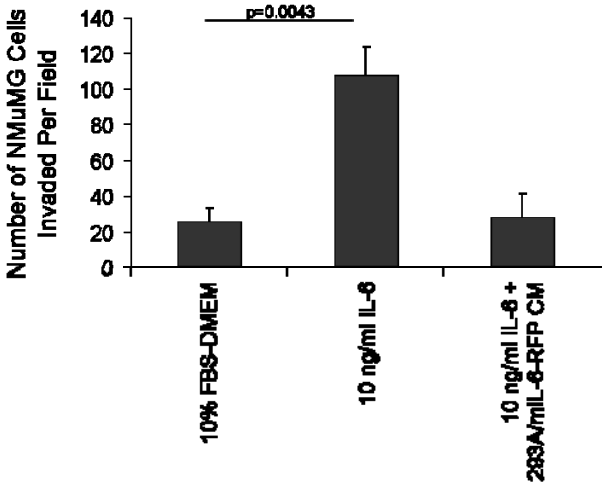

Figure 3-26. IL-6 treatment is sufficient to induce cellular invasiveness in NMuMG cells.

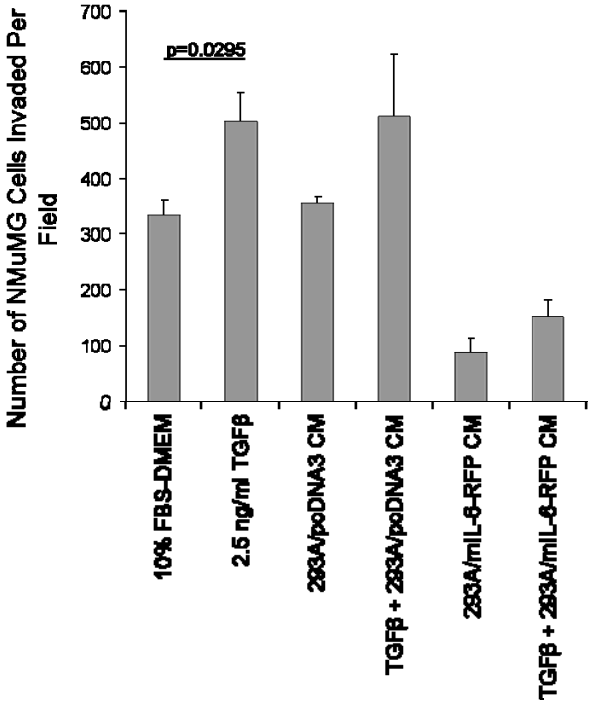

Figure 3-27. TGFβ treatment confers invasive potential on the otherwise nonmigratory NMuMG cell line.

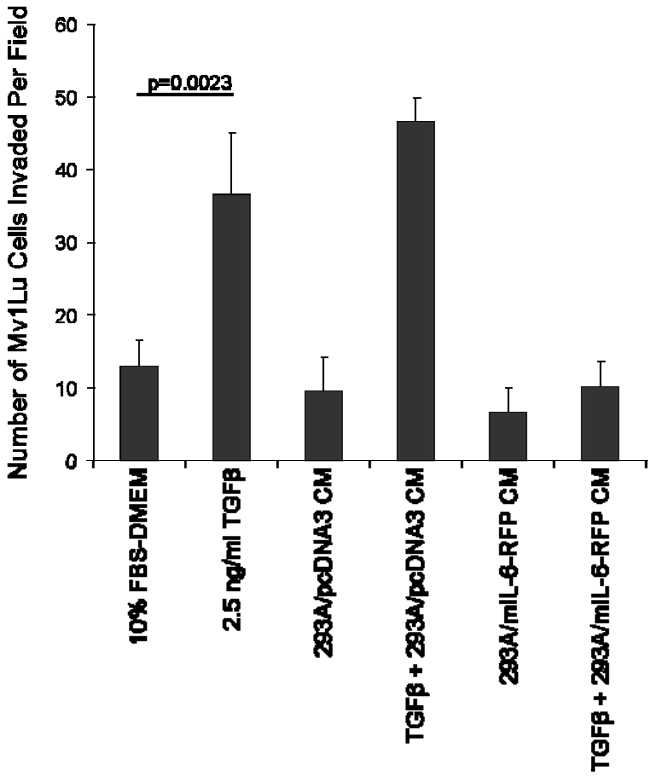

Figure 3-28. TGFβ treatment confers invasive potential on the otherwise nonmigratory Mv1Lu cell line.

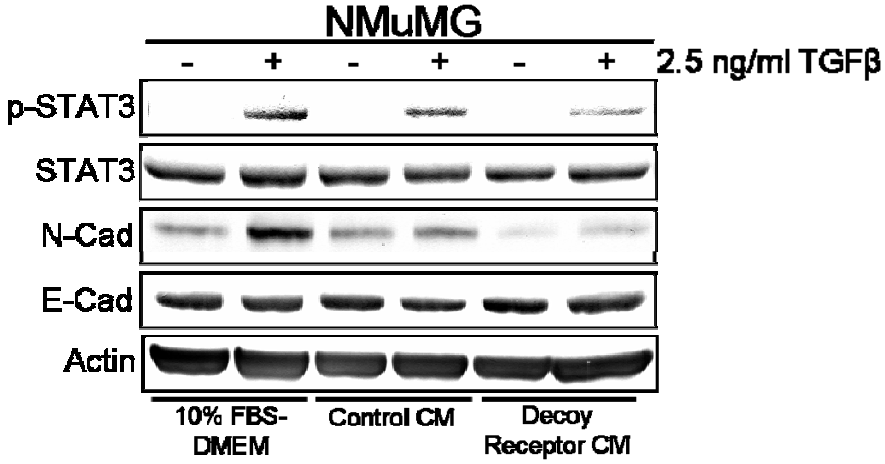

Figure 3-29. TGFβ induces upregulation of N-Cadherin that is IL-6 dependent.

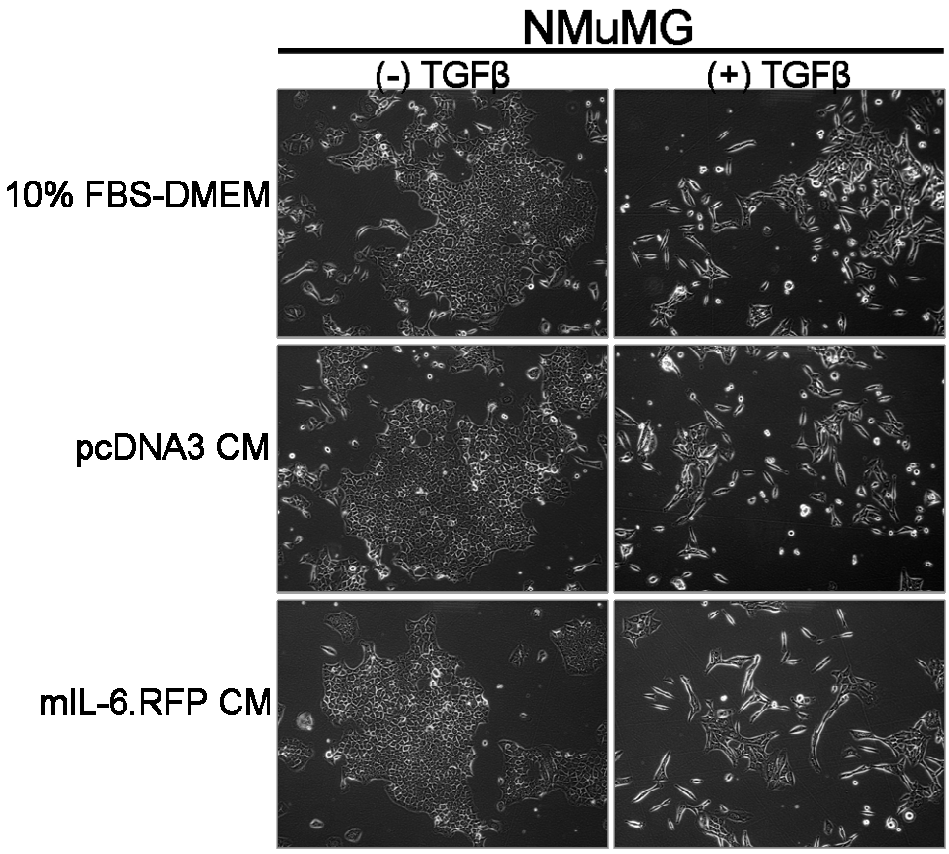

Figure 3-30. Inhibition of IL-6 function does not block the morphological changes associated with TGFβ-induced EMT.

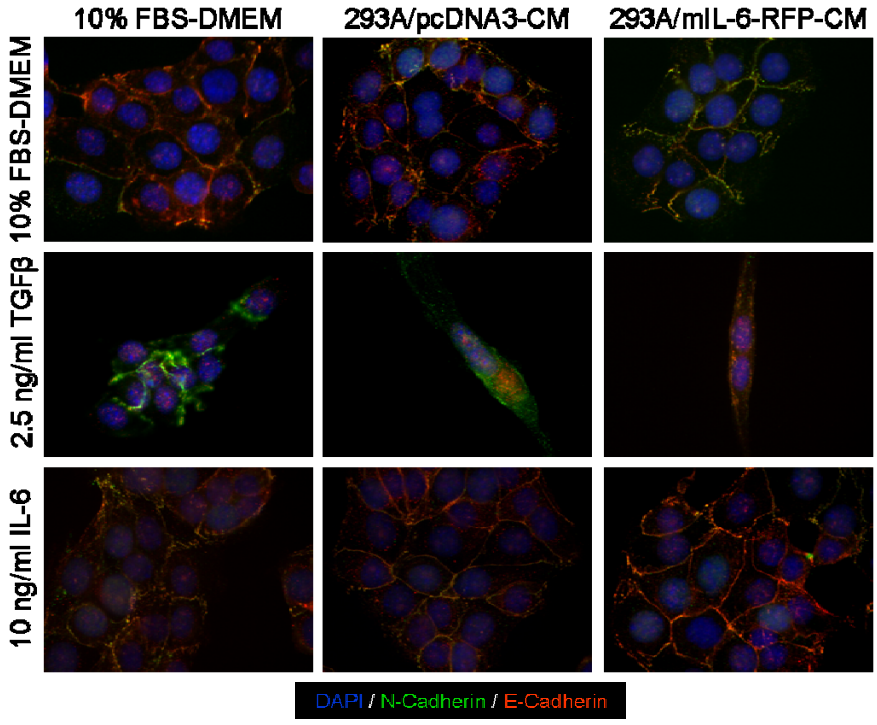

Figure 3-31. TGFβ induces N-Cadherin upregulation and E-Cadherin mislocalization in NMuMG cells; TGFβ stimulation of N-Cadherin upregulation is IL-6 dependent.

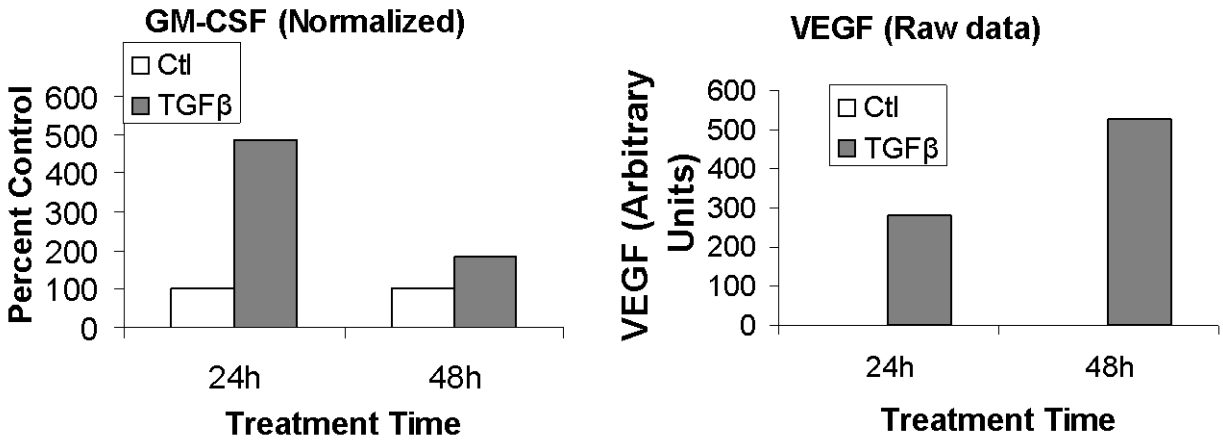

Figure 3-32. TGFβ induces secretion of Vascular Endothelial Growth Factor (VEGF) and Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF).

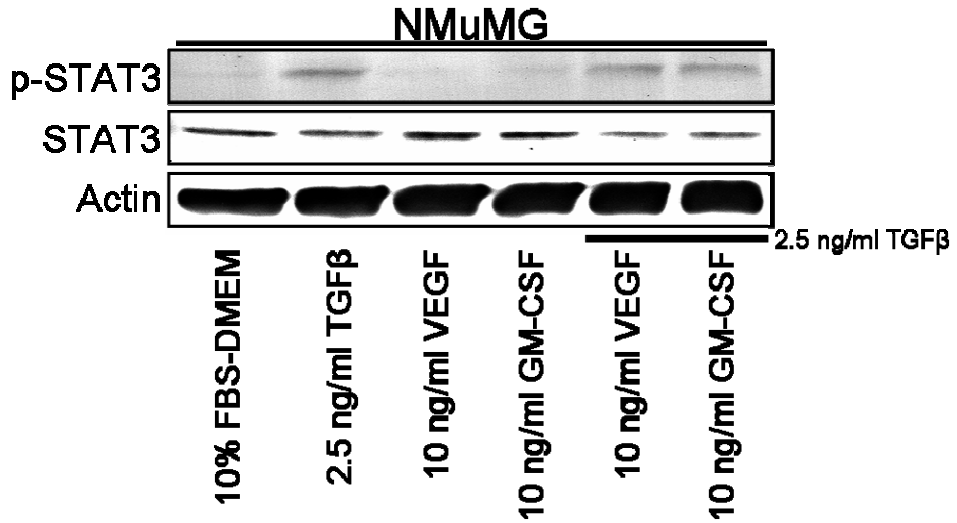

Figure 3-33. Exogenous VEGF and GM-CSF do not induce STAT3 tyrosine phosphorylation in NMuMG cells.

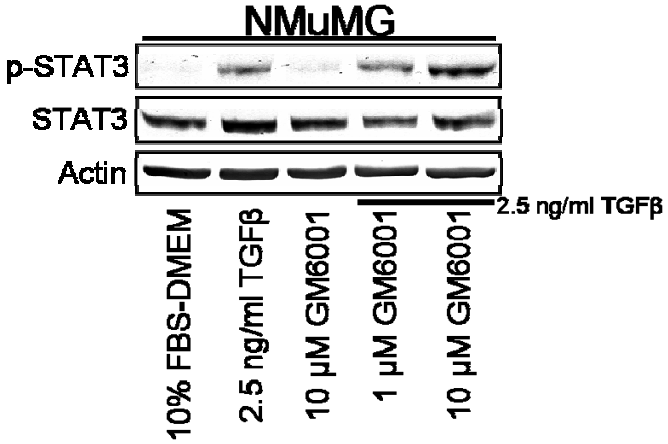

Figure 3-34. Broad-range inhibition of Matrix Metalloproteinases (MMPs) with the small molecule inhibitor GM6001 does not inhibit TGFβ stimulation of STAT3 tyrosine phosphorylation.

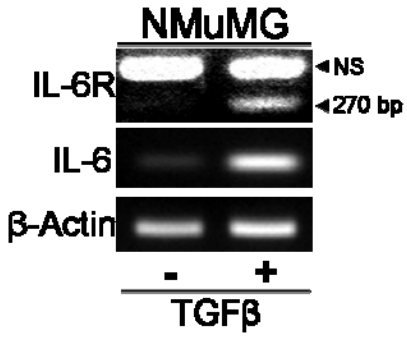

Figure 3-35. TGFβ treatment induces gene upregulation of the IL-6 receptor.

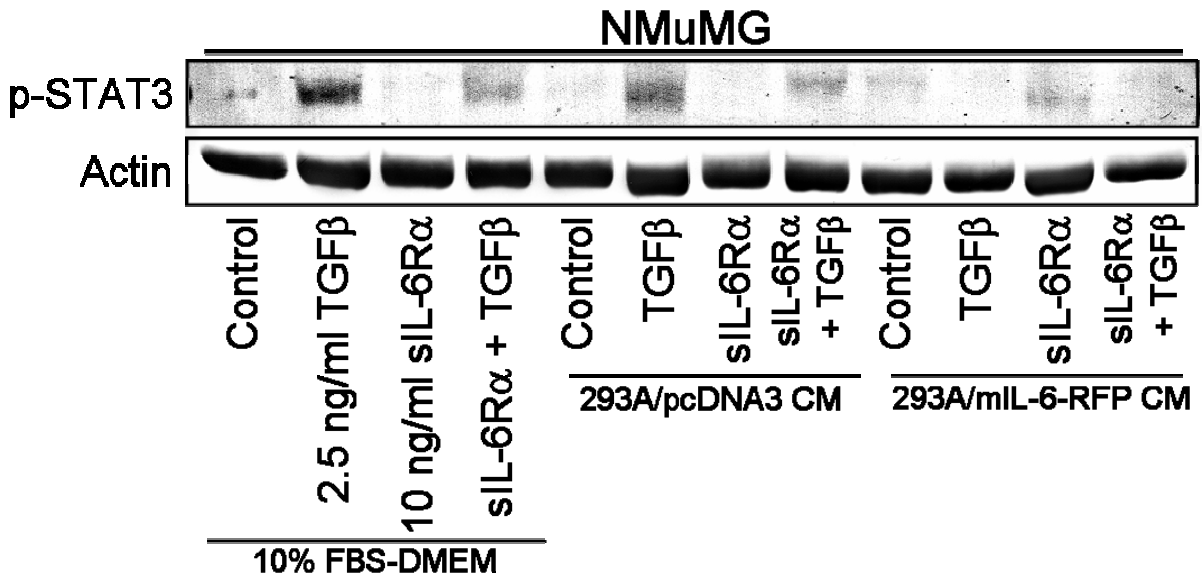

Figure 3-36. Exogenous soluble IL-6 receptor (sIL-6Rα) does not recapitulate TGFβ stimulation of STAT3 tyrosine phosphorylation.

CHAPTER 4
EVIDENCE FOR TGF-BETA INDUCTION OF STAT3 TYROSINE PHOSPHORYLATION IN HUMAN BREAST CANCER CELL LINES

Introduction

STAT3 is a transcription factor that has been implicated in various stages of the carcinogenesis process, including growth, inhibition of apoptosis, angiogenesis, invasion, and metastasis. Isolation of human breast cancer lines from harvested tumors is one way to study the mechanisms of cancer progression, as well as to develop new therapies without the use of expensive and time-consuming animal models. Recent studies have implemented molecular classification for breast cancer tumor types to classify tumors according to the molecular profile of cancer markers (342-344). For example, identification of triple-negative breast cancers—the basal-like subclassification—is significant because of the lack of expression of Her2, Progesterone Receptor, and Estrogen Receptor (342). These three receptors are the basis of the development of many chemotherapeutic agents and, in the absence of these receptors, few clinical agents are available for patients of triple-negative breast cancers. Therefore, identification of tumor markers can give clues as to the particular pathogenesis of breast tumors, as well as suggest specific therapies based on the tumor's molecular composition.

STAT3 is just such a marker. When phosphorylated on its Tyr (705) site, STAT3 correlates with poor patient prognosis (345-347), tumor grade (167, 348, 349), and metastasis (350) in various cancers. Significantly, many invasive breast cancer cell lines isolated from tumors display endogenous constitutive STAT3 phosphorylation, but so far the literature has outlined little reason for this phenomenon. Although two published studies (183, 184) indicate that sustained IL-6 secretion drives constitutive STAT3 phosphorylation, the mechanism was not fully characterized. Further, our data suggest that TGFβ contributes to secretion of IL-6 in the

NMuMG and Mv1Lu cell lines, which is a novel signaling mechanism not previously examined in detail.

Strikingly, the luminal-type human breast cancer cell line MDA-MB-361 (351) does not display constitutive STAT3 tyrosine phosphorylation, but the basal-like (351) breast cancer cell lines MDA-MB-231, BT549, and others do display this phosphorylation event. Using the MDA-MB-361 and MDA-MB-231 cell lines as a model for TGFβ maintenance of IL-6 secretion and, therefore, sustained STAT3 phosphorylation, we have uncovered evidence supporting the presence of this mechanism in human breast cancer lines.

To ultimately study the molecular basis behind human breast cancer treatment efficacies, one group's approach to understanding breast cancers was to categorize tumors according to molecular marker expression analysis. Sørlie et al. (342) sorted 65 normal and neoplastic breast tissue samples into the following categories: normal breast, luminal A, luminal B, ErbB2-overexpressing, and basal-like breast cancers. The human MDA-MB-231 and MDA-MB-361 cells lines have been described as basal-like and luminal, respectively (223). These cell lines were both derived from human breast tumors yet display varying levels of various epithelial and mesenchymal markers. Examples include Vimentin and Smooth Muscle Actin (SMA), both expressed by the basal-like MDA-MB-231 cells; the luminal marker E-Cadherin is expressed in MDA-MB-361 cells (223). A significant observation was made regarding the basal-like breast cancer subtype: basal-like breast cancer cell lines display a set of markers that had previously been defined as EMT markers (352). Molecular profiling of breast cancer cells and cell lines enabled such comparisons. Striking similarities may be drawn between EMT and basal markers, offering a functional explanation for breast cancer pathology. The properties of basal-like breast cancer cells and having undergone an EMT process both correlate with poor patient prognosis.

Taken together these observations suggest that there may be some overlapping etiological similarities between the process of EMT and the formation of basal-like breast cancers. Indeed, we have observed some data to this effect in our laboratory, the details of which are still being characterized.

Overall, due to the mechanism of TGFβ induction of STAT3 tyrosine phosphorylation that we characterized in the nontransformed mouse mammary NMuMG cell line, we had good reason to believe that this mechanism may be either inducible or already occurring in human breast cancer cell lines. In line with this reasoning we sought to stimulate TGFβ/IL-6/STAT3 signaling in the luminal MDA-MB-361 cell line, which displays no endogenous STAT3 tyrosine phosphorylation but responds to both IL-6 and TGFβ in terms of STAT3 phosphorylation. Similarly, we hypothesized that the MDA-MB-231 cell line, which exhibits constitutive STAT3 tyrosine phosphorylation, constantly expresses the TGFβ/IL-6/STAT3 signaling loop, and that this loop may be responsible in large part for the high degree of invasion observed in the MDA-MB-231 cell line.

Results

Constitutive STAT3 Tyrosine Phosphorylation and IL-6 Upregulation Varies Across Breast Cancer Cell Lines

We and others [Unpublished data, and (8)] have observed that different breast cancer cell lines display varying levels of constitutive STAT3 tyrosine phosphorylation. Thus far few (182) papers have investigated the significance of this observation. As indicated in Figure 4-1 and Figure 4-2, there appears to be a correlation between constitutive STAT3 tyrosine phosphorylation (Figure 4-1) and endogenous IL-6 upregulation (Figure 4-2) in these cell lines. The noninvasive mouse mammary NMuMG cell line and the human luminal MDA-MB-361 breast cancer cell line do not display STAT3 tyrosine phosphorylation in the absence of

stimulation, while the invasive and basal-like BT549, MDA-MB-231, and MDA-MB-468 breast cancer cell lines exhibit constitutive STAT3 tyrosine phosphorylation. Similarly, the MDA-MB-361 cell line displays low levels of endogenous IL-6 upregulation, while the MDA-MB-231 and BT549 cell lines produce high levels of IL-6 mRNA.

We chose to use the MDA-MB-231 and MDA-MB-361 cell lines as a model for TGFβ maintenance of STAT3 tyrosine phosphorylation. MDA-MB-231 cells display strong constitutive STAT3 tyrosine phosphorylation and IL-6 upregulation, while the MDA-MB-361 cell line does not. Therefore, we subsequently sought to demonstrate stimulation of the TGFβ-IL-6-STAT3 pathway in the MDA-MB-231 cell line and to induce these signaling events in the MDA-MB-361 cell line.

Conditioned Medium (CM) from Breast Cancer Cell Lines Variously Induces STAT3 Tyrosine Phosphorylation and STAT3-Dependent Transcription

Conditioned Medium (CM) was collected from the panel of breast cancer cell lines that was previously examined. The nontransformed R-1B cell line was used to study TGFβ Type I Receptor (TβRI) independent signaling mechanisms. We treated R-1B cells with the breast cancer CMs and examined STAT3 tyrosine phosphorylation by immunoblot analysis. The data indicate that there is a varying level of some cytokine or growth factor present in the CMs of the breast cancer cells (Figure 4-3), and that this cytokine is capable of inducing STAT3 tyrosine phosphorylation independently of TGFβ signaling. In addition, we cultured breast cancer cell lines in the presence or absence of a TGFβ Type I Receptor kinase inhibitor (TβRKI) and collected the conditioned medium. We transfected the m67-Luc STAT3 responsive luciferase construct into HepG2 cells due to this cell line's high transfection efficiencies and low endogenous STAT3 tyrosine phosphorylation. Treatment of the transfected HepG2 cells with the breast cancer CMs (from control or TβRKI samples) resulted in varying levels of STAT3

dependent luciferase activation (Figure 4-4, grey bars), corroborating the presence of some factor that is capable of inducing STAT3 dependent transcription. Further, TβRKI-CM of MDA-MB-231 and Tumor-Derived Fibroblast (TDF) cells induced lower levels of STAT3-dependent luciferase activity (Figure 4-4, open bars), indicating that whatever cytokine is present in the CM of breast cancer cells is dependent on TGFβ signaling. These data suggest that TGFβ is inducing expression of a growth factor or cytokine, which we hypothesized to be IL-6, that can induce STAT3 tyrosine phosphorylation. These data imply that this mechanism occurs in certain breast cancer cell lines, as we previously observed in the nontransformed mouse mammary NMuMG cell line.

A TGFβ Signaling Component Contributes to Maintenance of STAT3 Tyrosine Phosphorylation in the MDA-MB-231 Breast Cancer Cell Line

The basal-like human MDA-MB-231 breast cancer cell line displays constitutive STAT3 tyrosine phosphorylation, whereas the MDA-MB-361 human breast cancer line does not. Therefore, treatment with a TGFβ Type I Receptor Kinase Inhibitor (TβRKI) would indicate whether TGFβ signaling was involved with this phosphorylation event. As demonstrated in Figure 4-5, blockade of TGFβ Type I Receptor kinase activity markedly reduced endogenous STAT3 tyrosine phosphorylation, indicating involvement of the Type I TGFβ Receptor in the potential TGFβ-IL-6-STAT3 signaling loop. It is significant to note that MDA-MB-231 cells already display high levels of constitutive STAT3 tyrosine phosphorylation, and only marginal increases were observed with TGFβ or IL-6 stimulation (data not shown); increased phosphorylation may not be possible because STAT3 molecules are already maximally phosphorylated in MDA-MB-231 cells.

Interruption of the TGFβ-IL-6-pSTAT3 Loop Blocks STAT3 Phosphorylation, IL-6 Upregulation, and STAT3-Dependent Transcriptional Activity in MBA-MB-231 Cells

The IL-6 receptor fusion protein was used to inhibit IL-6 function in the MDA-MB-231 cell line, which produces constitutive IL-6 mRNA. Blockade of this protein would suggest proper IL-6 protein translation and regulation, which could contribute to this cell line's invasive and pro-growth properties. Indeed, blockade of IL-6 function with the IL-6 receptor fusion protein blocked both constitutive STAT3 tyrosine phosphorylation (Figure 4-6) and IL-6 upregulation (Figure 4-7), suggesting that not only is TGFβ involved, but that STAT3 phosphorylation is maintained by IL-6 secretion in this cell line. Therefore, these data point to a system in which TGFβ and STAT3 maintain IL-6 upregulation, which itself results in STAT3 tyrosine phosphorylation. In addition, transfection of a STAT3 responsive luciferase reporter gene (m67-luciferase) into the HepG2 cell line and treatment with MDA-MB-231 conditioned medium (CM) revealed that the MDA-MB-231 cells secrete a factor that induces STAT3-dependent transcription (Figure 4-8). Further, IL-6 inhibition with the mIL-6-RFP receptor fusion protein markedly reduced MDA-MB-231 induced STAT3 dependent transcriptional activation of the m67-luciferase reporter, indicating that IL-6 is secreted by MDA-MB-231 cells and is responsible for the STAT3-dependent transcription induced by CM from this cell line.

Abrogation of p-STAT3 Abolishes the Intrinsic Invasiveness of MDA-MB-231 Cells

Because the MDA-MB-231 cell line is intrinsically migratory and invasive (353), abrogation at any point in the TGFβ-IL-6-STAT3 signaling loop should inhibit this cell line's motile and invasive behavior. As shown in Figure 4-9, inhibition of TGFβ Type I Receptor kinase activity, Jak activity, IL-6 function, or STAT3 dimerization markedly reduced MDA-MB-231 cell invasion (Control versus TβRKI, $p<0.0001$, Student's *t*-test) through a Matrigel-coated

porous membrane. Taken together, STAT3 tyrosine phosphorylation by TGFβ-induced IL-6 upregulation is required for MDA-MB-231 cell motility and invasiveness.

Exogenous TGFβ Induces STAT3 Tyrosine Phosphorylation Through IL-6 Upregulation in MDA-MB-361 Cells

MDA-MB-361 cells, if all receptors and signaling are preserved, should result in STAT3 tyrosine phosphorylation upon treatment with exogenous TGFβ. Indeed, treatment with TGFβ strongly induced STAT3 tyrosine phosphorylation in the MDA-MB-361 cell line (Figure 4-10). Notably, IL-6 treatment caused the same effect: IL-6 treatment stimulated STAT3 tyrosine phosphorylation in MDA-MB-361 cells (Figure 4-10). Finally, treatment with TGFβ or IL-6 induced marked IL-6 upregulation in the MDA-MB-361 cell line (Figure 4-11), suggesting that this may be responsible for TGFβ induction of STAT3 tyrosine phosphorylation observed in this cell line (Figure 4-10).

Mouse Mammary Tumor-Derived Fibroblast (TDF) Conditioned Medium Recapitulates Exogenous TGFβ Treatment in MDA-MB-361 Cells

It has been demonstrated in many cancers, both histologically and pathologically, that tumor-associated fibroblasts are heavily associated with the nearby cancer cells (354). Mouse mammary derived tumor associated myofibroblasts (TDFs) were isolated from a basal-like breast cancer mouse model (276, 277) and have been shown to secrete TGFβ (276). We maintained the stable TDF cell line and collected conditioned medium (CM) periodically.

TDF-CM treatment induced robust STAT3 tyrosine phosphorylation in the human MDA-MB-361 breast cancer cell line (Figure 4-10). Strikingly, this effect was inhibited by Jak inhibition with the small molecule Jak inhibitor AG490, by Smad3 inhibition with the SIS3 small molecule inhibitor, and by inhibition of TGFβ receptor kinase activity with the small molecule kinase inhibitor TβRKI. This effect is significant because MDA-MB-361 cells can respond to TDF-CM by exhibiting STAT3 tyrosine phosphorylation, and because these tumor-associated

fibroblasts are likely a significant source of TGFβ, as we have observed to be the case in TDFs and which has been published for other fibroblastic cell lines (355).

TGFβ Treatment Confers the Invasive Phenotype on the Otherwise Noninvasive MDA-MB-361 Cell Line, Possibly Through TGFβ Induction of N-Cadherin Upregulation

The MDA-MB-361 cell line was plated with various treatments onto Matrigel-coated 8 μm-pore membranes and incubated with an FBS gradient for 72 h at 37 ºC. We observed that MDA-MB-361 cells are minimally invasive, but that invasiveness is greatly enhanced (Control versus TGFβ samples, p=0.0007, as measured by unpaired Student's *t*-test) with TGFβ treatment, and that this increase in invasion is markedly inhibited by IL-6 inhibition (Figure 4-12). It is important to note that MDA-MB-361 cells possess a high degree of homotypic cell-cell adhesion, with formation of colonies due to tight junction formation (356), partially explaining the low intrinsic invasiveness: whole colonies cannot squeeze through an 8 μm-pore as easily as a single, nonadherent cell. Along the same line of reasoning, we treated MDA-MB-361 cells with TGFβ or IL-6 and performed immunoblot analysis to examine N-Cadherin expression. We observed that TGFβ induced marked expression of N-Cadherin, as did IL-6 (Figure 4-13). Inhibition of IL-6 function was sufficient to abrogate N-Cadherin upregulation induced by both TGFβ and by IL-6 treatment. Therefore, because N-Cadherin has been demonstrated to be sufficient for cellular motility and invasion (250), we hypothesize that a major mechanism of TGFβ-induced invasion in MDA-MB-361 cells is due to IL-6 dependent N-Cadherin upregulation.

Finally, based on these data we constructed a model of TGFβ maintenance of IL-6 dependent STAT3 tyrosine phosphorylation in MDA-MB-231 cells (Figure 4-14), and a model for TGFβ induction of IL-6 dependent tyrosine phosphorylation in MDA-MB-361 cells (Figure 4-14). These two cell lines demonstrate that the TGFβ-IL-6-STAT3 signaling loop we observed

in nontransformed NMuMG cells is present or inducible in two human breast cancer cell lines, suggesting that these signaling events may be occurring in human breast cancer *in vivo*.

Conclusions and Discussion

In previous chapters we have outlined the significance of the TGFβ-IL-6-STAT3 mechanism in the greater context of carcinogenesis literature, and have presented data supporting this mechanism in nontransformed mouse mammary epithelial cells. However, the question of whether this mechanism operates in human breast cancer cell lines remained unanswered.

We have demonstrated in this chapter that not only do human breast cancer cell lines display varying levels of STAT3 tyrosine phosphorylation and IL-6 upregulation, but that the basal-like MDA-MB-231 breast cancer cell line displays TGFβ-dependent STAT3 phosphorylation and IL-6. The significance of this observation carries several implications. First, because TGFβ maintenance of IL-6 secretion and STAT3 tyrosine phosphorylation is occurring in this cell line, some genetic or perhaps epigenetic mechanisms are present that enable this mechanism to occur unabated. One possible explanation could be the downregulation of such STAT3 regulators as the SOCS and SHP2 proteins, which are downregulated or dysregulated in breast cancers and other cancers (310, 357-362). In an immune setting TGFβ can lead to SOCS3 downregulation (363), a potential mechanism that might be occurring in this system as well.

The second implication for the presence of the TGFβ-IL-6-STAT3 signaling loop in MDA-MB-231 cells concerns invasiveness. Each of these three molecules contributes to cellular migration and invasion in some system, but no studies have demonstrated that these are all involved in one pathway and are together responsible for inducing cellular invasiveness. To our knowledge this is the first study demonstrating that this mechanism confers cellular invasion on otherwise noninvasive cells, as we have shown with the MDA-MB-361 cell line.

Finally, the clinical implications are of some significance. Current TGFβ and IL-6 family inhibitors are in various stages of clinical trials; however, combination therapy of these agents with current chemotherapeutic agents that have been post-clinically demonstrated to act through the TGFβ signaling pathway holds encouraging promise for greater efficacy against cancers. Such combination regimens, once optimized, may potentially target tumor cells with greater selectivity, and therefore with fewer harmful and toxic side effects to the patient.

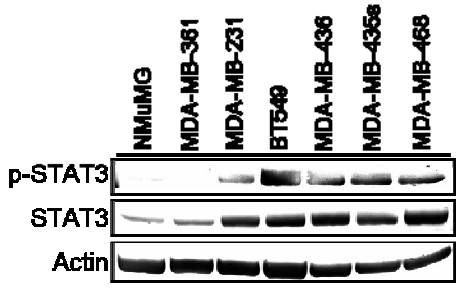

Figure 4-1. STAT3 expression and tyrosine phosphorylation vary across nontransformed epithelial and carcinoma cell lines.

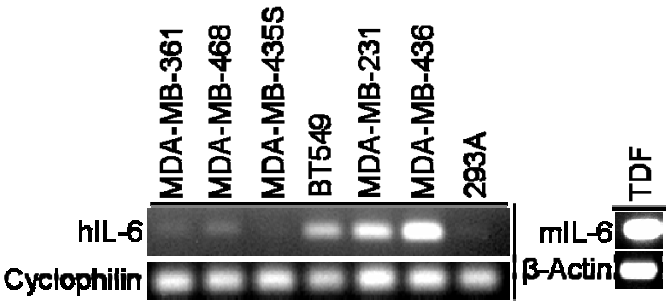

Figure 4-2. IL-6 upregulation appears to correlate with constitutive STAT3 tyrosine phosphorylation.

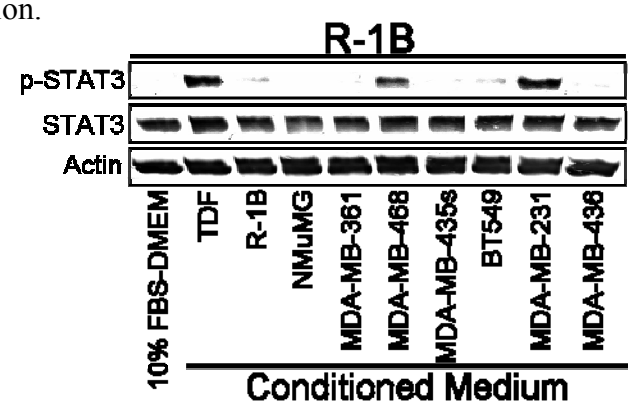

Figure 4-3. Treatment with conditioned medium from various breast cancer cell lines results in differential STAT3 tyrosine phosphorylation in a TβRI-inactive cell line.

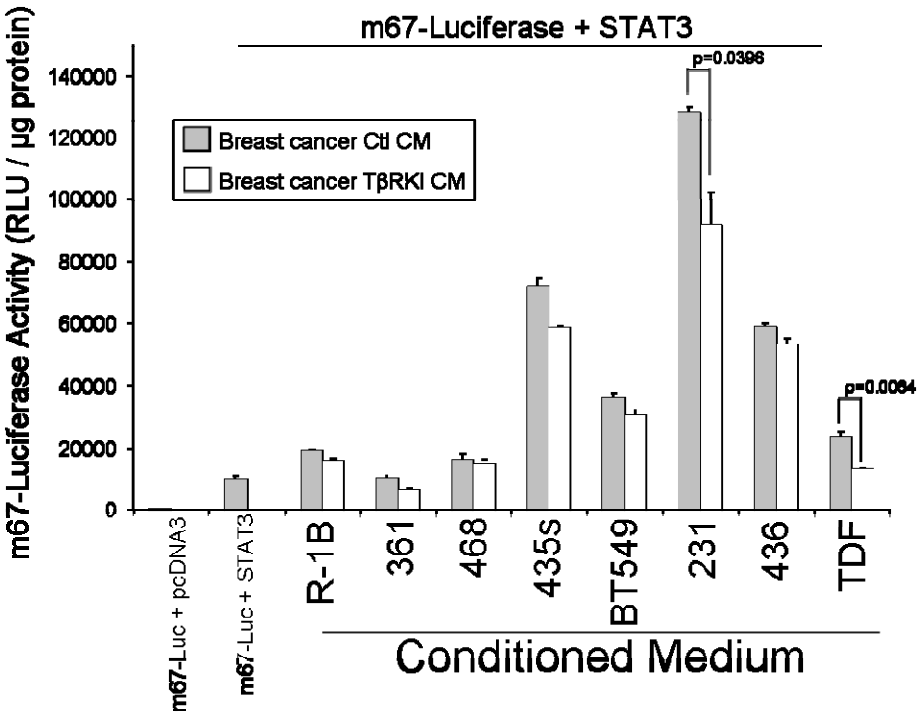

Figure 4-4. Treatment with CMs of various breast cancer cell lines induces varying levels of STAT3-responsive m67-luciferase activity that appears to be TGFβ dependent in MDA-MB-231 and Tumor-Derived Fibroblasts (TDFs).

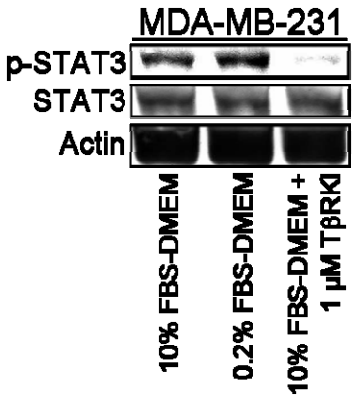

Figure 4-5. TGFβ drives maintenance of STAT3 tyrosine phosphorylation.

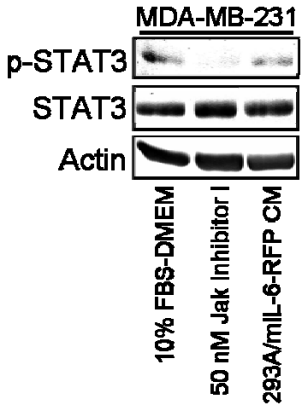

Figure 4-6. The invasive basal-like MDA-MB-231 breast cancer line exhibits constitutive STAT3 tyrosine phosphorylation that is TGFβ and IL-6 dependent.

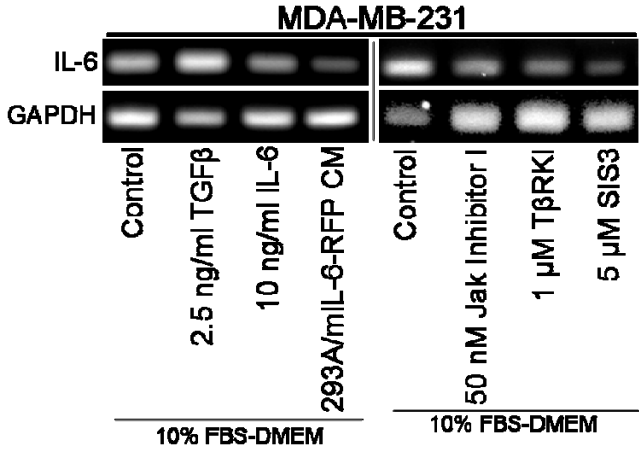

Figure 4-7. The MDA-MB-231 breast cancer line exhibits constitutive IL-6 upregulation that is TGFβ and IL-6 dependent.

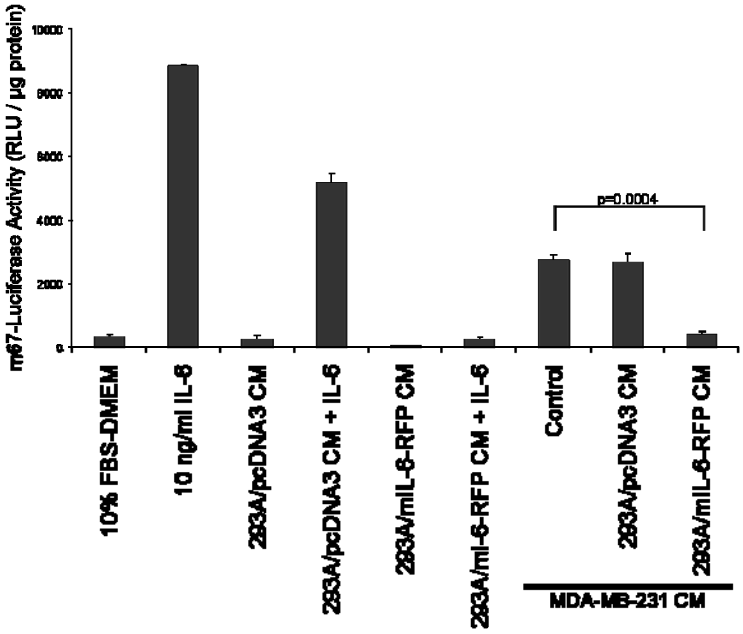

Figure 4-8. The invasive basal-like MDA-MB-231 breast cancer line displays STAT3 dependent luciferase transcription activity that is IL-6 dependent.

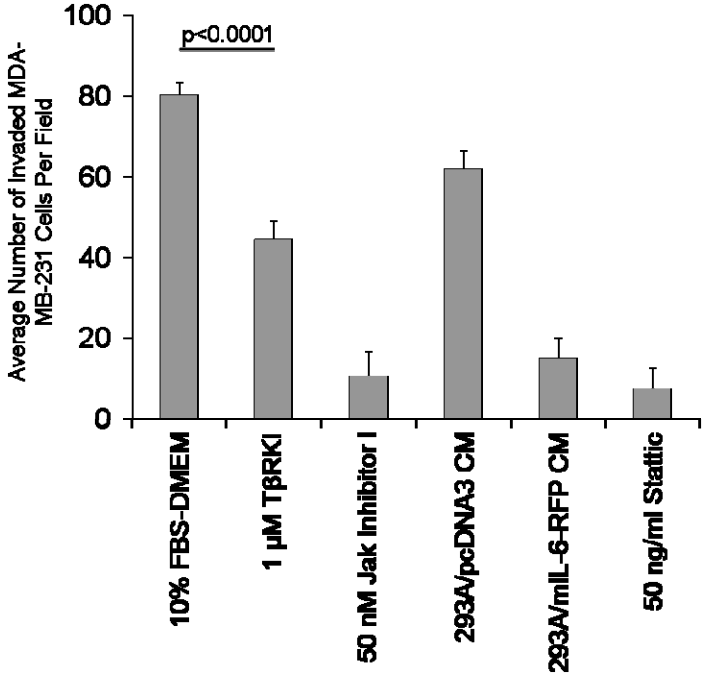

Figure 4-9. MDA-MB-231 invasion is TGFβ and IL-6 dependent.

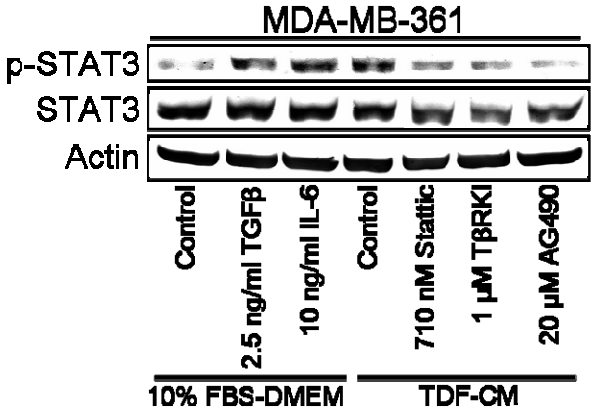

Figure 4-10. Tumor-derived fibroblast conditioned medium induces TGFβ and Jak-dependent STAT3 tyrosine phosphorylation in the MDA-MB-361 cell line.

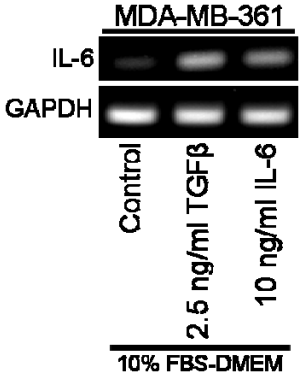

Figure 4-11. IL-6 and TGFβ treatment induce IL-6 gene upregulation in the MDA-MB-361 human breast cancer cell line.

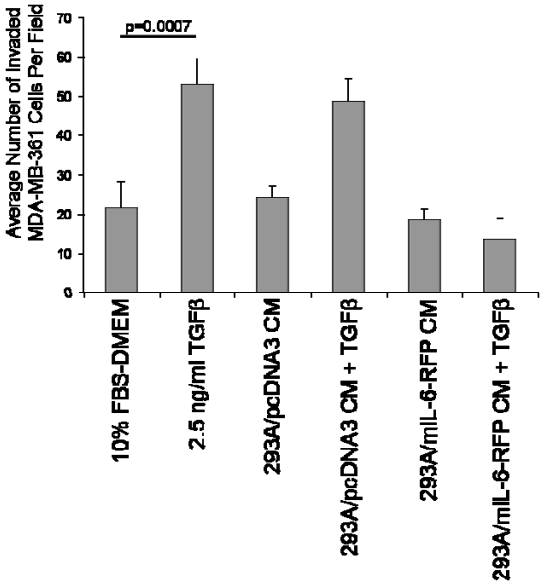

Figure 4-12. MDA-MB-361 cells acquire the invasive phenotype upon TGFβ treatment; this invasion is dependent on IL-6.

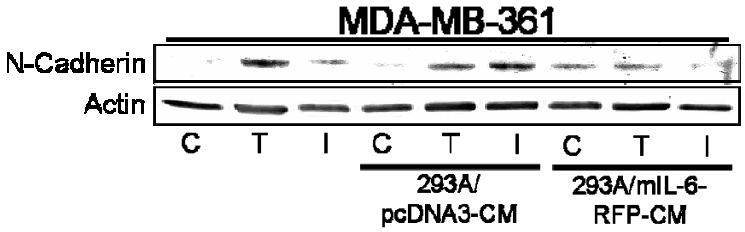

Figure 4-13. IL-6 and TGFβ treatment induces N-Cadherin upregulation in MDA-MB-361 cells; Blockade of IL-6 function blocks N-Cadherin upregulation.

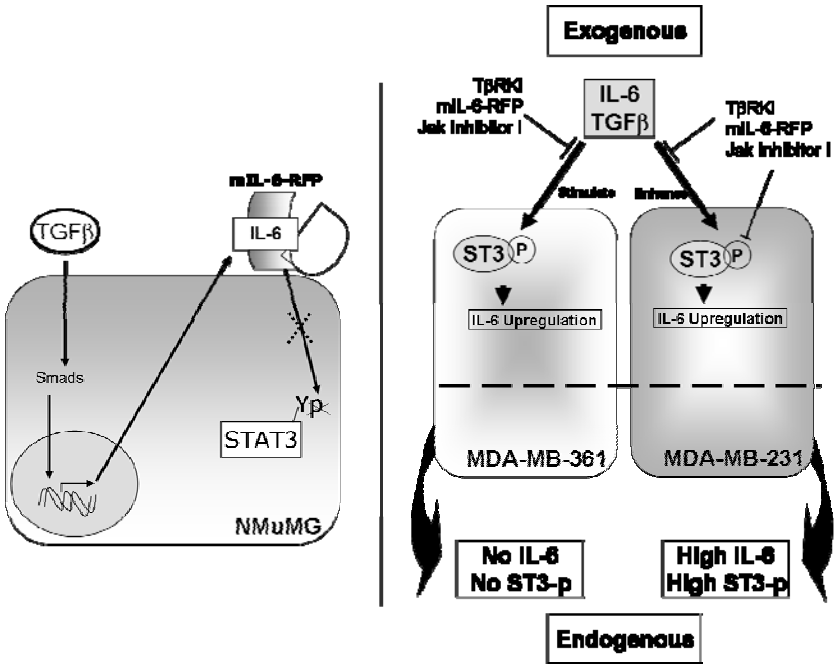

Figure 4-14. Proposed model for TGFβ and IL-6 dependent signaling in MDA-MB-231 and MDA-MB-361 human breast cancer cell lines.

CHAPTER 5
TOWARDS AN OVERALL CONCLUSION FOR TGF-BETA STIMULATION OF STAT3 TYROSINE PHOSPHORYLATION

Overall Findings and Significance

We have described herein a mechanism of TGFβ induction of STAT3 tyrosine phosphorylation. This phosphorylation is due to the upregulation of the cytokine IL-6; downstream effects of this upregulation include STAT3 tyrosine phosphorylation and acquisition of cellular invasiveness, potentially due to IL-6 dependent N-Cadherin upregulation. TGFβ induction and maintenance of STAT3 tyrosine phosphorylation through IL-6 upregulation is present in a basal-like model of breast cancer, the human MDA-MB-231 cell line. Abrogation of the TGFβ-IL-6-STAT3 signaling loop inhibits MDA-MB-231 invasion, and stimulation with TGFβ or IL-6 induces an IL-6 dependent increase in STAT3 tyrosine phosphorylation in the human MDA-MB-361 breast cancer cell line, which does not display endogenous STAT3 tyrosine phosphorylation.

There may be other aspects of TGFβ induction of STAT3 phosphorylation that are of importance: namely, the serum requirement of TGFβ stimulation of STAT3 phosphorylation, the exact role of the STAT3 Ser (727) site and its relationship to the tyrosine site and transcriptional significance, and whether LPA is involved in these mechanisms. It may be that these topics are artifacts of TGFβ-induced Epithelial to Mesenchymal Transition (EMT). In line with this hypothesis is our finding that IL-6 expression is responsible for N-Cadherin upregulation, possibly driving the invasive behavior that we have also demonstrated to be IL-6 dependent. In addition, IL-6 treatment is not sufficient to drive the morphological changes associated with EMT, while blockade of IL-6—and ostensibly N-Cadherin expression—is not sufficient to block the morphological changes to a fibroblastic phenotype. Together, these data suggest that N-Cadherin expression is dispensable for morphological changes associated with

EMT, and that it is insufficient to induce these changes; but that IL-6 induction of N-Cadherin expression appears to correlate with TGFβ induced invasiveness. Therefore, use of the IL-6R antibody Tocilizumab, or some other IL-6 targeted agent, might reduce the invasiveness of breast cancer cells *in vivo*. In reality, however, EMT is a complicated process and is only beginning to be well characterized using *in vitro* models. Many signaling pathways and molecules contribute to this program, which is inducible only in a few cell lines (59), and many researchers are attempting to investigate the process of EMT in the carcinogenic process *in vivo*.

Discussion and Future Work

We have described a mechanism of TGFβ induction of STAT3 tyrosine phosphorylation through IL-6 upregulation and secretion. Protein inhibition of IL-6 with the receptor fusion protein mIL-6-RFP blocks TGFβ stimulated STAT3 tyrosine phosphorylation. This new mechanism of STAT3 activation is significant because it appears to be necessary for cellular invasiveness of nontransformed mammary epithelial cells. Although others have demonstrated that TGFβ induces cellular invasion (84), the mechanism responsible for this effect was not well characterized. Further, TGFβ treatment induces EMT in NMuMG cells; however, IL-6 treatment does not recapitulate the mesenchymal phenotype, and is required for N-Cadherin expression and TGFβ stimulation of cellular invasion. TGFβ treatment in the presence of the IL-6 receptor fusion protein is sufficient to block N-Cadherin expression and invasion, but is not sufficient to inhibit the morphological changes associated with EMT.

There are several implications for these results. Others have demonstrated that N-Cadherin expression is sufficient for motility and invasion (250). However, our data suggest that N-Cadherin expression is not necessary for the morphological switch to a mesenchymal phenotype, but may mediate TGFβ-induced invasiveness.

We have demonstrated the presence of this pathway in a human breast cancer cell line, the basal-like (351) MDA-MB-231 cells. Significantly, this cell line displays constitutive STAT3 tyrosine phosphorylation and IL-6 upregulation, which both appear to be TGFβ dependent. Similarly, interruption of the TGFβ-IL-6-STAT3 pathway at any point resulted in abrogation of IL-6 upregulation and STAT3 tyrosine phosphorylation, and in marked inhibition of MDA-MB-231 cellular invasiveness.

The breast cancer subtypes, including basal-like breast cancers, are defined based on the gene expression profiles of breast tumors (342, 364). Significantly, basal-like breast cancers do not express Estrogen Receptor (ER), Progesterone Receptor (PR), or the Her2 receptor, and are referred to as Triple Negative breast cancers. Because many current breast cancer therapies target one of these three receptors, coupled with the fact that basal-like breast cancers predict a poor patient prognosis, basic understanding of the mechanisms of breast cancer development is more crucial than ever for the development of new therapies against this highly invasive breast cancer subtype. The overlap of basal-like breast cancer markers with EMT markers in breast samples has only strengthened the hypothesis that development of basal-like breast cancers and the EMT phenotype might share similar molecular underpinnings.

Future studies of STAT3 signaling will concentrate on the characterization of its activation upon TGFβ treatment: co-immunoprecipitation and complex formation experiments will elucidate the binding partners that are responsible for STAT3 tyrosine phosphorylation, which current data suggest are Jak kinases. Further, a relevant question that was examined in this study but not discussed is the role of the STAT3 Ser (727) site: much debate persists over what role this phospho-site plays in STAT3 gene transcription. Future work may implicate this phospho-site in, for example, transcription of certain sets of genes that are distinct from other

gene sets that are induced by tyrosine-phosphorylated STAT3. A large amount of work would be necessary to address this question, and is best served by future studies which lie beyond the scope of this project.

Other relevant and immediate questions linger concerning TGFβ stimulation of STAT3 tyrosine phosphorylation. There may be other cytokines or growth factors induced by TGFβ that are also, or instead, responsible for STAT3 tyrosine phosphorylation, rather than IL-6; one set of experiments that address this question is the use of IL-6 promoter luciferase reporter constructs that have various transcription factor binding sites mutated to abolish binding ability (available from the Belgian Coordinated Collection of Microorganisms, LMBP/BCCM). In this way examination of the promoter region, and therefore of the relevant structure-function relationships, can be attributable to Smad activity and not other factors. Removal of the relevant transcription factor binding site should produce no reporter activity, and should also abolish TGFβ induced STAT3 tyrosine phosphorylation in transfected cells. Ideally, data would reveal that Smad activity is responsible for IL-6 gene transcription, and Chromatin Immunoprecipitation (ChIP) analysis would corroborate identification of the Smad binding regions of the promoter. If Smads are causing activation of other transcription factors such as AP-1, then siRNA knockdown of the transcription factors might constitute an experiment addressing this possibility.

A second set of future experiments would address the question of EMT and N-Cadherin, and to what extent STAT3 and IL-6 are involved. For example, is IL-6 secretion—and therefore STAT3 tyrosine phosphorylation—necessary for only N-Cadherin expression in TGFβ-induced EMT, or is it just an artifact of the morphological changes and molecular upheaval that occurs? Further, are all these events present in tissues and neoplastic cells that undergo EMT *in vivo*? An

intriguing aspect of TGFβ-induced EMT is the role IL-6 and STAT3 tyrosine phosphorylation occupy in this complex process. Some studies have already identified a role for STAT5 in EMT (365). Because STAT5 and STAT3 share many oncogenic functions and both correlate with poor outcome in breast cancer patients (366, 367), it is plausible that STAT3 may participate in EMT as well. In line with this reasoning, our preliminary data have demonstrated that IL-6 is responsible for upregulation of the homophilic adhesion molecule N-Cadherin. However, blockade of IL-6 function, and therefore of N-Cadherin upregulation, does not inhibit the morphological changes associated with EMT, but does inhibit cellular invasiveness. Therefore, an interesting question is whether N-Cadherin expression itself is sufficient for cellular invasion in this cell line. Indeed, Johnson and Wheelock (230, 249-251, 255, 339, 368-380) have examined this process in great detail in various cell types, and have demonstrated that N-Cadherin, and not E-Cadherin, expression is essential for EMT induced motility and progression (250). However, it must be noted that among NMuMG cell lines, many clones exist that can greatly differ in characteristics and TGFβ responsiveness between different laboratories. Therefore, caution must be exercised in examination of similar processes in the same cell line among different clones. We have also observed that NMuMG cells lose TGFβ responsiveness over longer tissue culture passages. The late-passage NMuMG cells become spindle-shaped, begin to display endogenous STAT3 tyrosine phosphorylation, and appear to secrete TGFβ as measured by Pai-1 luciferase assays (data not shown), altogether resembling cells that have undergone EMT. Significantly, this brings up the question of whether more invasive and mesenchymal-type NMuMG cells can be induced by culturing cell populations over longer periods of time with many passages, or possibly with persistent TGFβ treatment until matricrine release. This model may be a more accurate representation of the cancer milieu, in which TGFβ

resides over long periods. Establishment of such a cell line would imply a correlation between earlier-passage cells, which are epithelial in behavior and marker expression, and low cellular invasiveness; and conversely, cells that have been cultured with TGFβ, or that have undergone an EMT-like process over long culture periods, become more mesenchymal-like and therefore more invasive. In this way it might be possible to draw more parallels *in vitro* between the EMT process and the basal-like phenotype, supporting gene array analysis data already published for several breast cancer cell lines (352).

Similarly, but perhaps of more clinical relevance, future studies will be required that concentrate on the role of TGFβ induced STAT3 activation in breast cancer development and invasion. An important link with human breast cancers, moreover, should be forged for proper examination of this mechanism. For example, biochemical analysis of human breast cancer tissues would suggest whether constitutive STAT3 phosphorylation is present; correlative analysis by immunohistochemistry would demonstrate a link between TGFβ and IL-6-Jak-STAT3 signaling. Tissue MicroArray (TMA) studies would facilitate this observation. Mouse studies that examine this problem would require genetic alterations of breast cancer cell lines. For example, the human breast cancer MDA-MB-231 cells might be altered to overexpress a dominant negative version of the Type I TGFβ Receptor (TβRI), the mIL-6-RFP receptor fusion protein, or to express siRNA directed to IL-6. These cell lines, together with the parental MDA-MB-231 cell line, would constitute a matched set of cell lines that could be used to examine tumor growth in nude mice. If, for example, mice injected with parental MDA-MB-231 cells reach a particular tumor volume sooner than mice injected with MDA-MB-231 cells engineered to overexpress IL-6 siRNA, then certainly tumor growth could be attributed at least in part to IL-6 secretion; and reintroduction of exogenous siRNA-resistant IL-6 would restore a pro-growth

phenotype. Conversely, blockade of TGFβ and IL-6 function in mice implanted with parental MDA-MB-231 cells would abrogate not only tumor growth, but also tumor invasiveness and metastatic ability. In addition, genetic analysis would be performed on the MDA-MB-361 cell line. For example, constitutive expression of TGFβ, IL-6, or a constitutively activated STAT3 construct (STAT3-C) should confer growth and invasive advantages, possibly through the upregulation of such factors as N-Cadherin. Further, mouse studies would confirm the presence of the TGFβ-IL-6-STAT3 signaling mechanism *in vivo*. Examples of these mouse studies that would elucidate the relationship between Tumor-Derived Fibroblasts (TDFs) and cancer cells would be to inject the mammary glands of nude mice with either MDA-MB-361 or Tumor-Derived Fibroblasts alone, or to co-inject these cell lines as a mixed population. If there is cross-talk between the two cell types *in vivo* there should be a marked increase in tumor growth and local invasion for co-injected sites compared to either site alone. After harvesting tumors we would examine the levels of STAT3 tyrosine phosphorylation in tumor cell extracts; MDA-MB-361 cells should respond to factors such as TGFβ and IL-6 that are secreted from the co-injected TDFs and are therefore present in the tumor microenvironment. In this way we would be able to quantify the growth advantage the MDA-MB-361 cells would have. Immunohistochemical analysis of tumor sections would allow identification of TDFs and cancer cells, and would reveal whether there is fibroblast infiltration into MDA-MB-361 tumors. If TDFs infiltrated amongst the cancer cells, this would suggest that the strong homotypic adhesions normally present in MDA-MB-361 cells are disrupted, perhaps due to IL-6 dependent N-Cadherin upregulation. Such tumor studies would yield valuable data about the *in vivo* implications of the TGFβ-IL-6-STAT3 signaling loop, and whether it confers heightened invasiveness and growth advantages in a mouse model.

Conclusions

Significant portions of the literature have focused on TGFβ and STAT3 as oncogenic factors that contribute to carcinogenesis and cancer invasiveness. However, few studies have focused on the interplay between these two pathways. Here we have presented data that suggest TGFβ induces STAT3 tyrosine phosphorylation through upregulation of the cytokine IL-6. Significantly, this occurs only with longer exposures to TGFβ, and the effect increases thereafter, presumably due to accumulation of IL-6 in the extracellular milieu. This may be a model of the chronic growth factor exposures that are present in precancerous lesions. In addition, these chronic exposures confer a migratory and invasive phenotype in the otherwise non-motile Mv1Lu cells. Significantly, these effects are highly dependent on intact TGFβ and IL-6-STAT3 signaling. Examination of these same signaling pathways in a human model of breast cancer, the basal-like MDA-MB-231 cell line, reveals the presence of this mechanism in this cell line. The prevalence of this mechanism is not known among breast cancer cell lines; however, we hypothesize that it is active in cell lines that express constitutively phosphorylated STAT3. Our hope is that this study and its associated publications will one day help drive the development of anticancer agents and therapeutic regimens that possess higher selectivity and efficacy in patients with fewer associated side-effects and less collateral damage.

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BIOGRAPHICAL SKETCH

Nicole Nadine Parker (née Teoh) was born in 1982 in Fort Wayne, Indiana, to William and Nihal Teoh. She grew up in various cities in Alabama, graduating from the Alabama School of Mathematics and Science in 2000. She attended McGill University and did her undergraduate research with Dr. Bertrand Jean-Claude in the Cancer Drug Research Laboratory. After graduating in December 2004 with a Bachelor of Arts in Economics with a minor in Biology, she moved to Gainesville, Florida and began working on osteoporosis in Dr. Tom Wronski's lab at the University of Florida. Upon her acceptance to the Interdisciplinary Program (IDP) in the College of Medicine, she received an Alumni Fellowship and has completed her graduate research under the mentorship of Dr. Brian Law. She plans to enter the field of science and medical writing and editing following her dissertation defense and subsequent degree conferral.