# TGF-β IN MAMMARY DEVELOPMENT AND TUMORIGENESIS

By

Brian Bierie

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Approved:

Dr. Harold L. Moses

Dr. Albert B. Reynolds

Dr. Neil A. Bhowmick

Dr. Joey V. Barnett

To my wife, family, colleagues and mentors

Thank you for the unwavering support

To those who are suffering and dying from cancer

I am doing everything in my power to help

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| | |
|---|---|
| Ab | Antibody |
| ALK | Activin-like kinase |
| ANOVA | Analysis of variance |
| APL | Acute promyelocytic leukemia |
| BMDC | Bone marrow derived cell |
| BME | 2-mercaptoethanol |
| BPH-1 | Benign prostatic hyperplasia epithelial cells |
| BRET | Bioluminescence resonance energy transfer |
| BSA | Bovine serum albumin |
| CAF | Carcinoma associated fibroblast |
| CBP | CREB binding protein |
| Ccl | C-C motif containing chemokine |
| Ccr | Ccl chemokine receptor |
| CD4 | Lymphocyte antigen CD4 |
| CD8 | Leu2 T-lymphocyte antigen |
| CD25 | Interleukin 2 receptor alpha |
| CD31 | Platelet/endothelial cell adhesion molecule |
| CD95 | Fas receptor |
| CD95L | Fas ligand |
| CDC42 | Cell division cycle 42 |
| cDNA | Complementary DNA |
| Ci | Curie |
| COX-2 | Cyclooxygenase-2 |
| CPM | Counts per minute |
| Cre | Cre recombinase |
| Ct | Threshold cycle |
| CTL | Cytotoxic T lymphocyte |
| CV | Coefficient of Variation |
| Cxcl | C-X-C motif containing chemokine |
| Cxcr | Cxcl chemokine receptor |
| DA | Dominant active |
| DAPI | 4',6-diamidino-2-phenylindole |
| DAXX | Death associated protein 6 |
| DCC | Deleted in Colorectal Carcinoma |
| DCIS | Ductal carcinoma in situ |
| DMBA | 7,12-dimethylbenz[a]anthracene |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | Dimethylsulfoxide |
| DN | Dominant negative |
| DNA | Deoxyribonucleic acid |
| DNIIR | Dominant negative type II TGF-β receptor |

| | |
|---|---|
| dnTβRII | Dominant negative type II TGF-β receptor |
| DPC4 | Deleted in Pancreatic Carcinoma; SMAD4 |
| EC | Extracellular domain |
| ECL | Enhanced chemiluminescence |
| ECM | Extra-cellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor ligand |
| EGFR | Epidermal growth factor receptor |
| ELISA | Enzyme linked immunosorbent assay |
| EMT | Epithelial to mesenchymal transition |
| ER | Estrogen receptor |
| EtOH | Ethanol |
| EVAc | Ethylene vinyl acetate copolymer |
| Fc | IgG fragment crystallizable |
| FGF-2 | Fibroblast growth factor 2 |
| FITC | Fluorescein isothiocyanate |
| FJP | Familial juvenile polyposis |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GS | Glycine serine rich domain |
| G-TsF | Glioblastoma-derived T cell suppressor factor |
| H&E | Hematoxylin and eosin |
| HGF | Hepatocyte growth factor |
| HIF | Hypoxia-inducible factor |
| HRP | Horse radish peroxidase |
| IACUC | Institutional Animal Care and Use Committee |
| IDC | Infiltrating ductal carcinoma |
| IF | Immunofluorescence |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IHC | Immunohistochemistry |
| JNK | c-Jun N-terminal kinase |
| K5 | Cytokeratin 5 |
| K8 | Cytokeratin 8 |
| kDa | Kilodalton |
| LAK | Lymphokine-activated killer |
| Lck | Lymphocyte-specific protein tyrosine kinase |
| LN | Lymph node |
| LOH | Loss of heterozygosity |
| LPS | Lipopolysaccharide |
| LTBP | Latent TGF-β binding protein |
| mAb | Monoclonal antibody |
| MAP3K1 | Mitogen activated protein kinase, kinase, kinase 1 |
| MC | MMTV-Cre |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MDSC | Myeloid derived suppressor cell |
| MEKK1 | Mitogen activated protein kinase, kinase, kinase 1 |

| | |
|---|---|
| MHC | Major histocompatibility complex |
| MIF-1 | Macrophage migration inhibitory factor-1 |
| MIN | Mammary intra-epithelial neoplasia |
| +MIN | Microsatellite instable |
| -MIN | Microsatellite stable |
| MIP-1 | Macrophage inflammatory protein-2 |
| MIP-2 | Macrophage inflammatory protein-2 |
| MISC | Myeloid immune suppressor cells |
| MKO | MMTV-Cre mediated knockout |
| MMP | Matrix metalloproteinase |
| MMTV | Mouse mammary tumor virus |
| MSI | Microsatellite instability |
| MSP-1 | Macrophage stimulating protein 1 |
| MST1 | Macrophage stimulating 1 |
| MUC1 | Mucin-1 |
| N+ | Lymph node positive |
| N- | Lymph node negative |
| NaCl | Sodium chloride |
| NaF | Sodium fluoride |
| NaOH | Sodium hydroxide |
| NBF | Neutral Buffered Formalin |
| NCI | National Cancer Institute |
| NIH | National Institutes of Health |
| NK | Natural killer cells |
| NKT | Natural killer T cells |
| NOD | Non-obese diabetic |
| Npt2b | Na–Pi type IIb co-transporter |
| Nude | Athymic Foxn1 null |
| OSM | Oncostatin-M |
| PAGE | Polyacrylamide gel electrophoresis |
| PAI-1 | Plasminogen activator inhibitor-1 |
| PAK | p21-activated kinase |
| PAR6 | Partitioning-defective protein 6 |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PDGF | Platelet-derived growth factor |
| PI3K | Phosphatidylinositol 3 kinase |
| PiMEC | Parity induced mammary epithelial cell |
| PML | Promyelocytic leukemia |
| PMN | Polymorphonuclear cell |
| PP2A | Protein phosphatase 2A |
| Prl | Prolactin |
| Ptgs2 | Cyclooxygenase-2 |
| PY | Polyoma virus middle T antigen |
| PyVmT | Polyoma virus middle T antigen |
| RNA | Ribonucleic acid |

| | |
|---|---|
| ROCK1 | Rho-associated, coiled-coil containing protein kinase |
| RT | Room temperature |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SAGE | Serial analysis of gene expression |
| SARA | Smad anchor for activation |
| SCC | Squamous cell carcinoma |
| SCID | Severe combined immunodeficient |
| SD | Standard deviation |
| SDF-1 | Stromal cell-derived factor-1 |
| SDS | Sodium dodecyl sulfate |
| SEM | Standard error of the mean |
| SMA | Smooth muscle actin |
| SMURF1 | Smad ubiquitination regulatory factor 1 |
| S-ODN | Soluble phosphorothioate oligodeoxynucleotide |
| SPCC | Spindle cell carcinoma |
| sRIII | Soluble truncated extracellular domain of TβRIII |
| SSC | Saline-sodium citrate |
| SSCP | Saline-sodium citrate phosphate |
| sTβRIII | Soluble TβRIII extracellular domain of TβRIII |
| TAK1 | TGF-β activated kinase 1 |
| TAM | Tumor-associated macrophage |
| TβRI | Type I TGF-β receptor |
| TβRII | Type II TGF-β receptor |
| TβRIII | Type III TGF-β receptor |
| TBS | Tris-buffered saline |
| TBST | Tris-buffered saline with TWEEN 20 |
| TEB | Terminal end bud |
| TGF-α | Transforming growth factor alpha |
| TGF-β | Transforming growth factor beta |
| TH2 | Type II T helper |
| TIL | Tumor-infiltrating lymphocyte |
| TM | Transmembrane domain |
| TNF | Tumor necrosis factor |
| TPA | 12-O tetradecanoyl phorbol 13-acetate |
| Tris | Tris(hydroxymethyl)aminomethane |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| uPA | Plasminogen activatorurokinase |
| VEGF | Vascular endothelial growth factor |
| vWF | von Willebrand Factor |
| WAP | Whey acidic protein |
| WC | WAP-Cre |
| WKO | WAP-Cre mediated knockout |

# CHAPTER I

# INTRODUCTION

## Overview

Transforming growth factor beta (TGF-β) signaling is known to regulate tumorigenesis, and in human cancer its signaling pathways are often modified during progression. Prior to initiation and early during progression, TGF-β acts upon the epithelium as a tumor suppressor, however at later stages it is often a tumor promoter. Recent studies addressing TGF-β mediated stromal-epithelial and host-tumor interactions have significantly improved our understanding related to the regulation of cancer. According to the current literature and experimental evidence, TGF-β is a potent mediator of carcinoma initiation, progression and metastasis through a broad and complex spectrum of interdependent interactions. Our current results, presented herein, primarily explore the mammary carcinoma cell response to TGF-β signaling and the impact on intrinsic, stromal-epithelial and host-tumor interactions during tumor progression *in vivo*. We have now shown that loss of the carcinoma cell TGF-β response results in carcinoma cell survival, increased smooth muscle actin positive stroma, tumor cell heterogeneity, inflammatory gene expression and inflammation that can promote tumor progression to metastasis. Further, our gene expression profiling results have been able to demonstrate a significant link between the loss of carcinoma cell specific TGF-β signaling and risk for relapse in human breast cancer.

## The TGF-β signaling network

The three TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3) signal through type I, II and III TGF-β receptors (TβRI, TβRII and betaglycan or endoglin respectively) to initiate signaling through downstream pathways (Lin et al., 1992; Wang et al., 1991). The TGF-β1 and TGF-β3 ligands, when expressed and activated through proteolytic cleavage or structural modification in the extracellular matrix or at the cell membrane, can bind TβRII with high affinity (Crawford et al., 1998; Lin et al., 1995; Munger et al., 1999; Yu and Stamenkovic, 2000). The active TGF-β2 ligand can only bind TβRII with high affinity in the presence of a TGF-β type III receptor (Lin et al., 1995; Moustakas et al., 1993). Prior to heteroligomerization, the TGF-β type I and type II receptors form homodimers in the endoplasmic reticulum and at the cell surface in the absence of ligand (Gilboa et al., 1998). TGF-β ligands preferentially bind to TβRII homodimers, and the ligand bound receptor complex has a high affinity for binding to and transactivating the TβRI receptors, resulting in an activated heteromeric signaling complex (Goumans et al., 2003b; Moustakas et al., 1993; Wrana et al., 1992).

Currently, seven type I and five type II TGF-β family receptors have been described. In most cell types, Alk-5 (*TGFβR1*) is the predominant TβRI activated by TGF-β through its cognate type II receptor, TβRII (*TGFBR2*). This interaction requires both extracellular and intracellular domains for signal transduction (Luo and Lodish, 1996). Alk-5 and TβRII proteins can also form active heteroligomeric complexes in the absence of ligand that are able to transduce basal signals when both receptors are coexpressed due to their intrinsic affinity for interaction (Feng and Derynck, 1996). In a limited subset of tissues, other TβRII–TβRI interactions have been reported. In

endothelial cells, Alk-1 and Alk-5 are both activated in response to TGF-β signaling through TβRII (Goumans et al., 2003b). In addition, another TβRI, Alk-2 (also known as TSK7L), has been directly implicated in signaling mediated by TGF-β *in vitro*, and in association with processes related to cardiac development *in vivo* (Olivey et al., 2006). Importantly, Alk-5 signaling results in activation of the transcriptional co-regulators Smad2 and Smad3, whereas Alk-1 or Alk-2 activate Smad1, Smad5 and Smad8 (Shi and Massague, 2003). These alternate receptor complexes are important to consider when interpreting results or designing therapeutic strategies involving modification of this pathway *in vivo*.

The functional TβRII–TβRI (Alk-5) heteromeric signaling complex is commonly associated with human cancer, and it regulates activation of downstream Smad dependent and Smad independent pathways (Figure 1) (Barrios-Rodiles et al., 2005; Derynck and Zhang, 2003; Moustakas and Heldin, 2005; Siegel and Massague, 2003). In response to activation through this complex the receptor associated Smads, Smad2 and Smad3, form homo- and heterotrimeric complexes involving the common mediator Smad4. These complexes then translocate to the nucleus where they regulate transcription (Feng and Derynck, 2005). In addition to the well characterized interactions known to regulate Smad activity, as exquisitely detailed in several review articles (Akhurst and Derynck, 2001; Derynck et al., 2001; Derynck and Zhang, 2003; Feng and Derynck, 2005; Massague, 2000; Siegel and Massague, 2003), one paper has recently shown that the Smad anchor for activation (SARA) requires a cytoplasmic promyelocytic leukemia (PML) isoform to bind Smad2 and Smad3 (Lin et al., 2004). This interaction was necessary for SARA to stabilize the Smad receptor interaction for activation by TβRI.

Primary fibroblasts that were deficient in expression of PML, similar to cells derived from patients with acute promyelocytic leukemia (APL), did not exhibit growth inhibition, cellular senescence or apoptosis in response to TGF-β stimulation (Lin et al., 2004).

In addition to the well characterized canonical Smad signaling pathways known to have a role in the regulation of cancer, Smad independent TGF-β regulated networks are currently known to include RhoA, Cdc42, Rac1, Ras, phosphatidylinositol 3 kinase (PI3K), protein phosphatase 2A (PP2A), mitogen activated protein kinase, kinase, kinase 1 (MAP3K1, also known as MEKK1), TGF-β activated kinase 1 (TAK1), death associated protein 6 (DAXX) and partitioning-defective protein 6 (Par6) (Barrios-Rodiles et al., 2005; Bhowmick et al., 2001; Chiu et al., 2001; Derynck and Zhang, 2003; Edlund et al., 2004; Moustakas and Heldin, 2005; Mulder and Morris, 1992; Ozdamar et al., 2005; Perlman et al., 2001; Petritsch et al., 2000; Shibuya et al., 1996; Siegel and Massague, 2003; Wilkes et al., 2003; Yamaguchi et al., 1995; Yi et al., 2005; Zhang et al., 2003). Although the cytostatic and apoptotic effects of Smad signaling are considerable when present (Akhurst and Derynck, 2001; Derynck et al., 2001; Derynck and Zhang, 2003; Feng and Derynck, 2005; Massague, 2000; Siegel and Massague, 2003), the balance and interplay between Smad dependent and independent signaling networks, ultimately control individual tumor cell autonomous and host-tumor interactions mediated by TGF-β in cancer (Figure 1). Further information regarding each of the Smad dependent and Smad independent pathways is given in (Akhurst and Derynck, 2001; Derynck et al., 2001; Feng and Derynck, 2005).

**Figure 1. Simplified diagram of TGF-β signaling.** Transforming growth factor-beta (TGF-β) ligands are activated in the extracellular matrix or at the cell membrane. The activated ligands bind the extracellular domain (EC) of type II TGF-β receptor (TβRII) homodimers with high affinity. The ligand-bound TβRII complex then binds and transactivates a type I TGF-β receptor (TβRI), which results in the phosphorylation of a glycine serine-rich region termed the GS box (GS). In carcinoma cells the predominant TβRI receptor is activin-like kinase 5 (ALK5). The activation of ALK5, a serine threonine kinase, results in the activation of downstream pathways. The level of signaling through each downstream pathway is context and cell type dependent. The resulting net activation of downstream pathways in each cell type determines the response to TGF-β *in vivo* and *in vitro*. Significant roles for canonical Smad signaling in the TGF-β pathway have been identified that mediate the TGF-β-dependent regulation of tissue homeostasis and cancer. The Smad independent networks are known to include CDC42, cell division cycle 42; DAXX, death-associated protein 6; EMT, epithelial–mesenchymal transition; MAP3K1, mitogen-activated protein kinase, kinase, kinase 1; PAK, p21-activated kinase; PAR6, partitioning-defective protein 6; PI3K, phosphatidylinositol 3-kinase; PP2A, protein phosphatase 2A; ROCK1, Rho-associated, coiled-coil containing protein kinase 1; SMURF1, Smad ubiquitination regulatory factor 1; TAK1, TGF-β-activated kinase 1; TM, transmembrane domain.

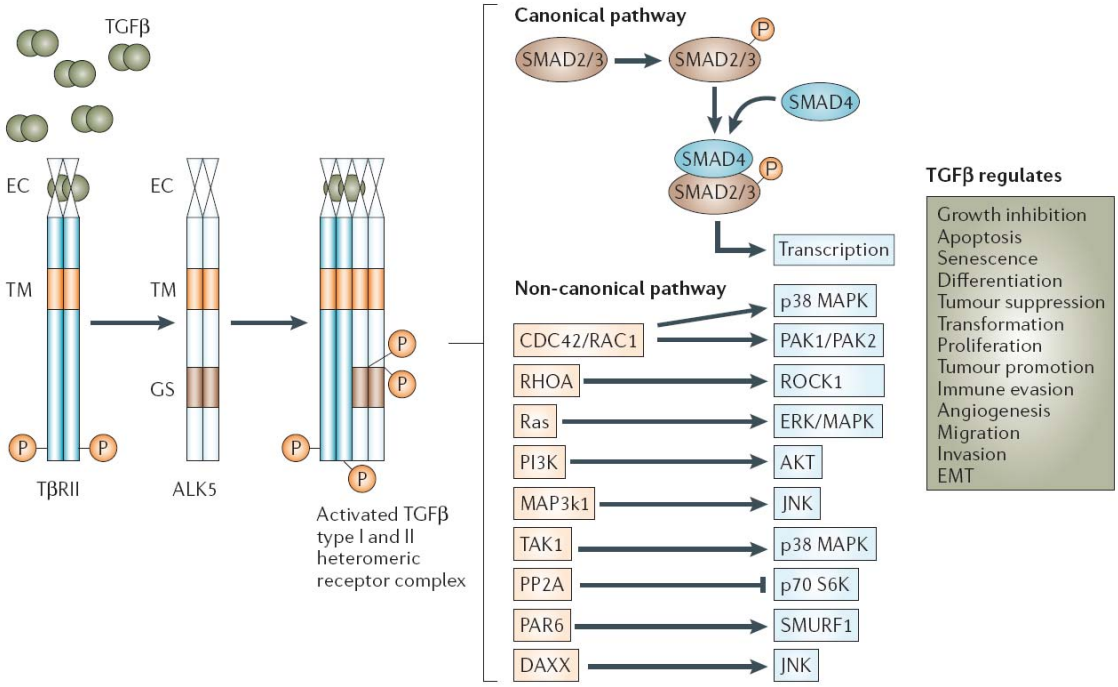


**Figure 1: Simplified diagram of TGF-β signaling.**

## General features of TGF-β signaling associated with cancer

The tumor microenvironment is a broad term that refers to all of the cells and signaling factors present in any specific cancerous lesion. Matrix and stromal signals are essential for the regulation of cancer, and many of these interactions involving TGF-β have been thoroughly reviewed (Bhowmick et al., 2004b; de Caestecker et al., 1997; de Caestecker et al., 2000; De Wever and Mareel, 2003; Derynck et al., 2001). The presence of a diverse population of cell types responding to TGF-β in the tumor microenvironment creates a complex milieu of factors that together regulate cancer initiation, progression and metastasis.

In cancer, TGF-β1 is upregulated to a greater extent than either TGF-β2 or TGF-β3 (Derynck et al., 1987; Dickson et al., 1987), and as a result TGF-β1 has been the focus for most of the cancer related studies to date. Initial observations in epithelial cells demonstrated that TGF-β1 could induce growth inhibition suggesting a role in tumor suppression (Moses, 1985; Tucker et al., 1984). Previously, it was shown that TGF-β could transform normal fibroblasts (Moses et al., 1981; Roberts et al., 1981). TGF-β signaling has since been implicated in an array of cell type and tissue specific responses. It is now generally accepted that TGF-β signaling has multiple roles in epithelial cell types, from which malignant carcinomas arise, ranging from arrest of cell growth (Moses, 1985; Pierce et al., 1993; Tucker et al., 1984; Wakefield and Sporn, 1990) to enhancing migration (Dumont and Arteaga, 2000; Dumont et al., 2003) and initiating changes in cell morphology (Bakin et al., 2002; Bakin et al., 2000; Bhowmick et al., 2001; Brown et al., 2004; Miettinen et al., 1994; Oft et al., 1998; Oft et al., 1996; Xie et al., 2003; Zavadil et al., 2001). These predominant features have been the focus for many studies

investigating TGF-β signaling that encompass tumor cell autonomous and host-tumor dependent interactions.

In cancer, TGF-β will often suppress early tumorigenesis and later enhance tumor progression (Derynck et al., 2001; Pasche, 2001; Roberts and Wakefield, 2003). The specific response to TGF-β during tumor progression can be attributed to both independent and interrelated factors including changes in receptor expression, availability of downstream signaling components, evasion of the immune response, stimulation of inflammation, presence of local and systemic factors (autocrine, endocrine, paracrine, juxtacrine or matricrine interactions) and the recruitment of cell types that lead to an advantage in tumor growth or promote angiogenesis. Recently, several studies have illustrated new cell autonomous effects and relationships between the stromal and epithelial compartments mediated by TGF-β that can significantly contribute to cancer initiation and progression. These observations may provide new avenues for exploration or modification of the TGF-β signaling cascade in translational cancer research with further experimental analysis.

There are several aspects of cellular communication that must be considered when addressing TGF-β related signal transduction *in vivo*. Epithelial cells communicate with each other through secretion of soluble factors, junctional complexes or interactions with matrix and basement membrane substrates that promote signal transduction and liberation of additional signaling factors. Fibroblasts communicate in their microenvironment mainly through the secretion of soluble factors that interact with neighboring cells and deposition of extra-cellular matrix (ECM) components. Fibroblasts also possess the ability to transdifferentiate in response to TGF-β, producing a myofibroblast that is able

to communicate with the tumor microenvironment through secretion of soluble factors and modification of the ECM. Epithelial cells, fibroblasts and myofibroblasts are each able to produce and respond to cytokines and growth factors. Ablation of TGF-β signaling in these cell types from the tumor microenvironment can have profound cell autonomous, local and systemic consequences. Consequently, fibroblast and epithelial cell autonomous signaling in addition to the resulting stromal-epithelial interactions are essential for regulation of cancer initiation and progression. Disrupting the cell autonomous signaling or stromal-epithelial interactions can lead to both developmental defects and carcinoma *in vivo*.

## Regulation of the TGF-β pathway in human cancer

TGF-β signaling has both positive and negative effects on cancer depending on the cell type and context in which a mutation, amplification or deletion occurs. Genetic alterations that have been identified in the TGF-β pathway include TGF-β ligand and receptor mutation, deletion or amplification. In addition, mutations and altered expression profiles for SMAD family members, which are central TGF-β signaling components, have been observed in human cancer (Levy and Hill, 2006).

TGF-β mis-regulation has a significant impact on tumor progression and patient prognosis. Increased serum levels of TGF-β1 associated with a mutation in the *TGF-β1* gene, for example, have been associated with a lower incidence of breast cancer (Ziv et al., 2001). This correlation suggests that the TGF-β1 ligand promotes tumor suppression during the early stages of initiation and progression. Complimentary observations have

shown that low levels of TβRII expression correlate with an increased risk of breast cancer (Gobbi et al., 1999). In immunohistochemical studies on frozen sections it was shown that out of 45 invasive breast carcinoma cases only 2% stained positive for TβRII while TGF-β1 was present in 67% of the cases examined (de Jong et al., 1998b). When TβRII was present it was correlated areas of higher microvessel density (de Jong et al., 1998a). Interestingly, endogenous overexpression of TβRII in the stromal compartment of human breast cancer correlated with a poor prognosis for the patient (Barlow et al., 2003). This critical observation suggested that in addition to tumor cell autonomous effects of TGF-β signaling, stromal cell stimulation by TGF-β may contribute to regulation of the adjacent epithelial cell population in human tumors. In contrast to the role of tumor suppression during tumor initiation and early progression, TGF-β1 may actually promote cancer progression in later stages. TGF-β is expressed at high levels during these late stages, as illustrated in many studies including observations correlating with advanced disease progression in human colorectal cancer (Tsushima et al., 1996). Further, in prostate cancer, TGF-β1 upregulation has been associated with angiogenesis, metastasis and poor patient prognosis (Wikstrom et al., 1998). This particular study, involving 73 patients, also demonstrated that the loss of TβRII expression in tumors from patients with elevated TGF-β1 correlated with a significant decrease in survival compared to the control population (Wikstrom et al., 1998). These combined results suggest that TGF-β is able to mediate cell autonomous, local and systemic responses that together regulate initiation, progression and prognostic outcome in human cancer.

In human cancer *TGFBR2* is often mutated and the conserved mutations in a large percentage of the cases lead to pathway inactivation (Grady et al., 1999). The *TGFBR2*

gene is commonly the target for both microsatellite instable (+MIN) and microsatellite stable (-MIN) mutations in cancer (Akhurst and Derynck, 2001; Grady et al., 1999). *TGFBR2* mutations occur frequently in colon cancer (+MIN 30%; -MIN 15%), gastric cancer (+MIN 30-80%; -MIN <5%), glioma (+MIN 70%; -MIN <5%), non-small cell lung cancer (+MIN 75%; -MIN <5%) and pancreatic cancer (Akhurst and Derynck, 2001). Point mutations occur in the *TGFBR2* gene as shown in cells isolated from human tumors (Lucke et al., 2001). Further, inherited and spontaneous colon tumors have been associated with mutation of a microsatellite repeat region in the *TGFBR2* gene (Grady, 2004; Markowitz et al., 1995). This region in the *TGFBR2* gene, encoding a portion of the extracellular domain, is often mutated in mismatch repair deficient colon cancers and results in a truncated protein that lacks function. In addition to colon cancer, *TGFBR2* microsatellite region mutations have been noted in other mismatch repair deficient tumor tissues including the breast (Seitz et al., 2003). Compared to the prevalence of microsatellite region mutations in colon cancer the observed mutations in liver, pituitary gland, myelodisplastic syndrome, endometrial or breast cancers are relatively rare (Kretzschmar, 2000; Takenoshita et al., 1998). In human colon cancer it has also been shown that 55% of the microsatellite stable cases harbor mutations distal to the *TGFBR2* receptor that effectively block signaling through this pathway (Grady et al., 1999). The conservation and frequency of mutations observed for *TGFBR2* in human cancer indicates that a selective advantage exists for inactivation of this pathway during tumorigenesis.

Signaling mediated by TGF-β involves activation of a number of direct downstream targets including MEKK1, TAK1, MAPK, PI3K, Ras, RhoA, PP2A and

SMADs that have been investigated in cancer (Bakin et al., 2000; Derynck and Zhang, 2003). The only downstream targets that are thought to be specific for the TGF-β family are the SMADs. In pancreas and colon cancer, regions of the human locus *18q21* encoding SMADs 2 and 4 are often mutated or lost completely (Blobe et al., 2000; de Caestecker et al., 2000; Derynck et al., 2001; Eppert et al., 1996; Hahn et al., 1996; MacGrogan et al., 1997; Massague, 2000; Massague and Chen, 2000; Schutte et al., 1996; Takagi et al., 1998; Thiagalingam et al., 1996). In pancreas cancer 90%, of the cases demonstrate a loss at *18q21* which includes both the *DPC4* (*Deleted in Pancreatic Carcinoma*; *SMAD4*) and *DCC* (*Deleted in Colorectal Carcinoma*) genes (Hahn et al., 1996). The deletion in pancreatic cancer was further isolated to *18q21.1* which includes *DPC4*, and excludes *DCC* as the target for mutation. The loss of *SMAD4* eliminates the classic Smad2/3/4 heteromeric complexes that have been implicated in a large number of TGF-β dependent transcriptional regulatory complexes. *SMAD4* mutations are rare but present in the breast, and *SMAD2* mutations have been detected at low levels in colon, head and neck, and lung carcinomas (Kretzschmar, 2000). *SMAD3* mutations have not been identified in correlation with any specific cancer, however the level of expression may be altered during progression as observed in human gastric or extravillous trophoblast cancer (Han et al., 2004; Xu et al., 2003). Gastric cancer cells that lacked *SMAD3* were not growth inhibited by TGF-β, and upon re-introduction of *SMAD3* again demonstrated growth inhibition (Han et al., 2004). Loss of *SMAD3* expression in choriocarcinoma cells has been linked to downregulation of TIMP-1, and this may allow the enhanced activity of MMPs classically identified to have a role in tumor invasion (Xu et al., 2003). In another informative study, reduction of SMADs 2 and 3 from human

breast cancer cell lines resulted in enhanced tumorigenesis with a reduction in metastasis (Tian et al., 2003). Implications from this data and many other related studies clearly indicate that SMAD family mutations and mis-regulation can contribute to the progression of cancers from which they were derived.

In the tumor microenvironment, many factors can contribute to the presence of an active TGF-β signal including transcriptional, translational or protein processing differences between adjacent cell types (Mizoi et al., 1993). This is an interesting issue, since many studies assume that the presence of TGF-β mRNA means that the ligand will be translated and processed properly. This is not always the case for adjacent cells within the tumor microenvironment, since subsequent translation or processing of the protein product may be hindered in a specific cell type sub-population within a tumor. An interesting study published over a decade ago used an immunoelectron microscopy approach to address the production of pro-TGF-β1 and the latent TGF-β binding protein (LTBP) products in gastric carcinoma associated cell types. The electron microscopy identified differences in the intracellular distribution of these products between stromal and epithelial gastric cancer-derived cell types. In the cancer stromal cells pro-TGF-β1 was located in the rough endoplasmic reticulum and perinuclear cisternae. Conversely, in the cancer-derived epithelium pro-TGF-β1 was dispersed throughout the cytoplasm indicating that epithelial cell intracellular transport may be defective. In addition, LTBP was only detected in fibroblasts and smooth muscle cells derived from the gastric carcinomas and found in the ECM surrounding these cells (Mizoi et al., 1993). These results indicate that in some tumors and cell types expression of bioactive and functional

TGF-β depends on cell specific factors that include a combination of transcriptional, translational and post-translational processes for functional activity *in vivo*.

**The role for TGF-β signaling during tumor initiation, progression and metastasis**

Over the past two decades, it has been clearly shown that TGF-β can delay early tumorigenesis in mouse models that recapitulate human cancer. In one of the first studies conducted *in vivo*, transgenic mice overexpressing bioactive TGF-β1 under control of the mouse mammary tumor virus promoter/enhancer (MMTV-TGFβ1) demonstrated a tumor suppressive role for this pathway when bred to the transgenic MMTV-TGFα (transforming growth factor alpha) mouse model of mammary tumorigenesis (Pierce et al., 1995). In this study, it was also shown that the MMTV-TGFβ1 mice had a delay in tumorigenesis when treated with 7,12-dimethylbenz[a]anthracene, a potent chemical carcinogen. In a similar study it was shown that TGF-β1 was able to suppress tumorigenesis in the MMTV-c-neu transgenic mouse model of mammary tumorigenesis (Siegel et al., 2003). Conversely, the loss of one TGF-β1 allele lead to enhanced tumorigenesis in response to carcinogen treatment, and *TGF-β1* gene disruption alone was able to enhance colon carcinoma formation (Engle et al., 1999; Tang et al., 1998).

Several studies have shown that expression of dominant negative TβRII constructs in mice led to enhanced mammary tumorigenesis either in the presence of carcinogens or without (Amendt et al., 1998; Bottinger et al., 1997b; Gorska et al., 2003; Siegel et al., 2003). Further, squamous cell carcinomas result from conditional deletion of Smad4 from mouse mammary epithelial cells *in vivo* (Li et al., 2003). Results obtained from the conditional ablation of Smad4 in mammary epithelium, confirm the

importance of signaling through the TGF-β/Smad pathway during tumor initiation and progression suggested in earlier reports of enhanced colon tumorigenesis using Smad4 null mouse models (Takaku et al., 1998; Takaku et al., 2002; Taketo and Takaku, 2000). Further, these effects were not limited to the mammary gland and colon since dominant negative TβRII expression in the lung and skin also resulted in enhanced carcinogen induced tumorigenesis (Amendt et al., 1998; Bottinger et al., 1997a).

Many recent studies have been designed to address the issue of micro-environment *versus* epithelial cell autonomous TGF-β mediated effects in cancer using dominant active (DA), dominant negative (DN) or conditional knockout TBRII receptors *in vivo*. Most of the available transgenic and knockout models are unique in the precise aspects of TGF-β signaling they address. Due to the unique nature of each mouse model, though individually informative, they often should not be directly compared. In one such study, DA and DN TBRII constructs were crossed with two mutant MMTV-ErbB2 transgenic lines that constitutively signal through only Grb2 (YB) or Shc (YD) pathways (Siegel et al., 2003). The data indicated that signaling through the TβRII receptor results in an increase of tumor latency with a reduction in metastasis. The results from this study further indicated that overexpression of a dominant active type I TGF-β receptor (TβRI) resulted in a delay of tumorigenesis with an increase in metastasis. In another related study, epithelial cell specific ablation of TβRII in mammary tissues from mice expressing the MMTV-PyVmT (polyoma virus middle T antigen) transgene resulted in decreased mammary tumor latency with an increase in pulmonary metastases (Forrester et al., 2005). Both studies indicate that TGF-β is an early tumor suppressor, but the results are in direct opposition regarding the role for TGF-β during metastasis. The differences

between these studies may indicate, amongst other possibilities, that the specific epithelial cell autonomous tumor promoter driving each individual carcinoma can contribute to a different net response elicited by TGF-β *in vivo*. In addition, another significant difference was the expression of a dominant negative construct *versus* total ablation of the receptor. Both of these models in addition to other related studies, despite their differences, indicate that modification of epithelial cell autonomous TGF-β signaling can significantly influence tumor progression and metastasis.

Cell culture and xenograft methods have also been widely used to analyze the TGF-β contribution to epithelial cell autonomous regulation of tumor progression and metastasis. One of the first studies to clearly suggest a role for TGF-β during tumor progression and metastasis, demonstrated enhanced invasion *in vitro* and metastasis *in vivo* after treating adenocarcinoma cells with TGF-β (Welch et al., 1990). The enhanced invasion *in vitro* and metastasis *in vivo* was thought to be partially attributed to TGF-β dependent upregulation of type IV collagenase activity (Welch et al., 1990). It is now known that many factors contribute to this process in response to TGF-β stimulation. Epithelial cells can be induced to increase migration and invasion in response to TGF-β, while inhibition of systemic TGF-β has been shown to reduce the number of metastases from MMTV-ErbB2 overexpressing tumors (Ueda et al., 2004; Yang et al., 2002a). The conditional expression of TGF-β1 in MMTV-PyVmT tumor cells also promoted metastasis without having an effect on primary tumor proliferation or size. Inhibition of TGF-β1 in MMTV-PyVmT tumor cells reduced basal cell motility, survival, anchorage-independent growth, tumorigenicity, and metastases. The results suggested that the changes in metastasis were epithelial cell autonomous (Muraoka-Cook et al., 2004).

These results are consistent with those previously obtained in which the soluble Fc:TβRII fusion protein (Fc:TβRII) was used to block TGF-β signaling in transgenic and transplantable models of breast cancer metastases (Muraoka et al., 2002). Further, expression of a soluble truncated extracellular domain of TβRIII (sRIII) in cells derived from a pleural effusion of human breast cancer (MDA-MB-231) prevented metastasis when compared to controls after injection into nude mice (Bandyopadhyay et al., 1999). The expression of sRIII reduced the available TGF-β ligand in cultured medium and it was suggested that the resultant reduction in TGF-β signaling mediated the change in metastatic potential (Bandyopadhyay et al., 1999). Together these results help to substantiate and confirm numerous postulates suggesting that there is a delicate balance between the net signal transduction driving a tumor and the response elicited by subsequent or concurrent TGF-β stimulation *in vivo*.

## Shifting the paradigm: identification of TGF-β dependent stromal-epithelial interactions within the tumor microenvironment

Carcinoma suppression mediated by TGF-β is not limited to carcinoma cell autonomous signaling. Recent results that highlight stromal-epithelial crosstalk in the regulation of cancer have demonstrated that the stromal fibroblast response to TGF-β signal transduction can be important for suppression of tumorigenesis in adjacent epithelium (Bhowmick et al., 2004a). The role for TGF-β mediated tumor suppression in this context involved the negative regulation of secreted factors such as hepatocyte growth factor (HGF), macrophage stimulating protein 1 (MSP1) and TGF-α that are overexpressed by fibroblasts when TβRII expression has been conditionally ablated

(Bhowmick et al., 2004a; Cheng et al., 2005). The first study to report this regulatory role for TGF-β *in vivo*, involved conditional ablation of *exon 2* from *Tgfbr2* specifically in fibroblasts (Bhowmick et al., 2004a). This resulted in prostatic intraepithelial neoplasia and invasive squamous cell carcinoma of the forestomach that occurred with 100% penetrance. In this model, HGF was upregulated and complementary activation of the HGF receptor c-Met was detected in tissues where TβRII had been ablated, implicating this paracrine signaling network as a potential mechanism for regulation of carcinoma (Bhowmick et al., 2004a). It has now been shown, using fibroblasts derived from these mice, that paracrine HFG, MSP1 and TGF-α signaling results in activation of c-Met, RON, ErbB1 and ErbB2 in adjacent epithelium when combined with carcinoma cells under the subrenal capsule (Cheng et al., 2005). When compared with control fibroblasts, those lacking TβRII were able to promote invasion of adjacent carcinoma cells *in vivo*. In addition, tumors produced from carcinoma cells grafted with fibroblasts lacking TβRII were more proliferative, exhibited a higher degree of angiogenesis and a decreased rate of apoptosis when compared to tumors produced from carcinoma cells grafted with control fibroblasts. Further, inhibition of HGF, MSP1 or TGF-α signaling in conditioned medium derived from TβRII deficient fibroblasts, has been shown to attenuate the increased proliferation and migration of carcinoma cells treated with the conditioned medium *in vitro* (Cheng et al., 2005). The expression of these ligands, in particular TGF-α and HGF, suggest that stromal-epithelial interactions have a significant role in the regulation of adjacent carcinoma initiation and progression. TGF-α and activated erbB2 have been widely used to induce mammary tumors in mice (Guy et al., 1992b; Matsui et al., 1990). In addition, HGF expression in the mammary gland has

recently been shown to result in lobular-alveolar hyperplasia and subsequent adenocarcinoma (Gallego et al., 2003). The current results *in vivo*, suggest that ablation of TGF-β signaling within stromal fibroblasts can result in activation of paracrine signals that are able to act upon adjacent epithelial cells to promote tumor initiation and progression. Together, these results indicate that distinct TGF-β responses mediated by stromal fibroblasts can regulate carcinoma initiation and progression of adjacent epithelium *in vivo* and *in vitro*.

The contribution of stromal carcinoma associated fibroblast signaling in the tumor microenvironment has been known for many years (Barcellos-Hoff and Ravani, 2000; Hayward et al., 2001), and we are now beginning to understand some of the mechanisms governing these interactions. It is clear that there are differences between fibroblast cell populations with respect to regulation by TGF-β, however it is not completely clear which factors regulate the differential responses to TGF-β stimulation. It is likely that the distinct molecular profile, and microenvironment associated with an individual fibroblast population (for example prostate stromal fibroblast *versus* mammary stromal fibroblast), determines the response to TGF-β stimulation *in vivo*. The concept of unique signaling in alternate fibroblast cell populations, has been addressed through global mRNA expression analyses, that indicated that distinct molecular profiles could be used to identify the tissue from which individual fibroblast cell populations were derived (Chang et al., 2002). The alternate transcriptional signatures observed for fibroblasts derived from different areas of the body has been termed positional memory. The effect of positional memory on TGF-β signaling may partially explain why carcinomas were observed specifically in the prostate and forestomach of mice expressing a fibroblast

specific ablation of TβRII (Bhowmick et al., 2004a). A recent study that further demonstrated this type of differential response to TGF-β stimulation, contrasted fibroblasts derived from fetal and adult tissues. In fetal fibroblasts, stimulation with TGF-β resulted in growth inhibition, while in adult fibroblasts stimulation resulted in enhanced proliferation (Giannouli and Kletsas, 2005). This study clearly demonstrated that individual subpopulations of fibroblasts initiate unique molecular programs in response to TGF-β stimulation. Together, these results indicate that TGF-β may act to suppress carcinoma promoting factors in some fibroblasts, but this mechanism for tumor suppression may be dependent on the distinct molecular profile of each individual fibroblast cell population *in vivo*.

Modification of the TGF-β pathway in stromal fibroblasts has been shown to regulate tumorigenesis in adjacent human mammary epithelium. This is important, since many of the previously described systems involved modified mouse models and it was not clear if this type of stromal-epithelial interaction would have an impact on the regulation of human mammary epithelium. However, this has now been addressed in a mouse-human hybrid system (Kuperwasser et al., 2004). Human fibroblasts and mammary epithelial cells used for these experiments were derived from reduction mammoplasty. The human fibroblasts were used to "humanize" NOD/SCID mouse mammary fat pads before transplantation of the human mammary epithelial cells, thus allowing the normal growth of human mammary epithelial cells in the mouse. In these experiments, genetic changes in the stromal fibroblasts used for colonization of the humanized mouse mammary fat pads could alter the phenotype of the implanted epithelial cells. When normal mammary epithelial cells were not co-injected, the growth

of the mammary epithelial cells had a predisposition toward varying levels of hyperplasia through carcinoma. The abnormal lesions may have been due to precancerous alterations in the mammary epithelial cells that were suppressed by the normal stromal fibroblasts. Further, when TGF-β1 expression was increased in humanized fat pad fibroblasts, additional human mammary epithelial cell derived lesions were observed. Interestingly, the lesions in the TGF-β1 overexpressing humanized stromal fibroblast mice resembled lesions obtained from mice with humanized stromal fibroblasts overexpressing HGF (Kuperwasser et al., 2004).

Stromal-epithelial interactions derived from the fibroblast compartment that are dependent upon TGF-β signaling can clearly regulate initiation and progression of tumors in adjacent epithelia. However, it has now been shown that this type of stromal-epithelial interaction is not limited to fibroblast derived regulation of tumor suppression. TGF-β signaling in T cells can regulate carcinoma in adjacent epithelium (Kim et al., 2006). Specifically, ablation of Smad4 in T cells can result in adjacent gastrointestinal carcinoma. In this study, the Lck and CD4 promoters were used to inactivate the Smad4 gene in mouse T cells using Cre-LoxP technology. At nine months of age the small and large intestines were significantly thickened in all of the animals lacking Smad4 expression in their T cell populations. In addition, 25% of the animals demonstrated rectal prolapse. The lesions observed in the thickened intestines were primarily linear sessile and peduncular polyps. Histological analyses revealed that the lesions in this model had a large stroma rich mixed mononuclear stromal cell infiltrate. It was also shown that the deletion of the Smad4 signaling in T cells resulted in enhanced plasma cell activation and TH2 cytokine production (including interleukins IL-4, IL-5, IL-6, IL-9

and IL-13). In this model the heterozygous loss of Smad4 signaling produced results similar to the homozygous loss of this protein *in vivo*. Interestingly, it was also shown in this study that deletion of Smad4 signaling in the gastrointestinal epithelium alone, using two different promoters to drive Cre expression, did not result in gastrointestinal carcinoma (Kim et al., 2006). The results associated with T-cell signaling obtained in this experimental system may also have clinical relevance. In human familial juvenile polyposis (FJP), an autosomal dominant disorder that is associated with hamartomatous polyps and gastrointestinal cancer, *SMAD4* germline mutations are observed with a penetrance of approximately 50% (Howe et al., 1998; Howe et al., 2002). Results obtained in the mice with deficient Smad4 signaling in T cells suggest that the T cell populations in human FJP patients may be contributing to their carcinoma associated disease initiation and progression (Kim et al., 2006). Further, these results together with those previously described in fibroblasts (Bhowmick et al., 2004a; Cheng et al., 2005), suggest that TGF-β signaling in stroma cell populations can potently regulate carcinoma initiation and progression in adjacent epithelium.

In addition to direct fibroblast associated stromal-epithelial interactions, TGF-β signaling has in some systems been associated with transdifferentiation of fibroblasts to myofibroblast cell types. The transdifferentiation if present may be important since the myofibroblast cells could provide a new subset of cytokines and growth factors that contribute to tumor progression. Tumor-derived TGF-β can cause fibroblast to myofibroblast differentiation, and the resulting myofibroblasts can produce smooth muscle actin, cytokines, proteases and matrix components. HGF is an example of a factor secreted by cells following TGF-β-induced myoepithelial differentiation that can

contribute to enhanced migration of the tumor epithelial cells (Lewis et al., 2004). This suggests a system wherein tumor cells may produce TGF-β that can cause myofibroblast differentiation, followed by myofibroblast secretion of a tumor enhancing factor. Tenascin-C and HGF produced by myofibroblasts have been shown to act synergistically to promote invasive tumor progression. The synergistic activity of tenascin-C and HGF was shown to involve signaling through RhoA and Rac to promote invasion (De Wever et al., 2004a). The invasive potential of the myofibroblast compartment has been implicated in the invasive potential of the adjacent cells within the tumor microenvironment. TGF-β is able to upregulate N-cadherin at the tips of the filopodia in myofibroblasts and this contributes to their relative invasive potential. Activation of c-Jun N-terminal kinase (JNK), seems to regulate TGF-β-mediated invasion and expression of N-cadherin. This regulation in myofibroblasts was shown through pharmacological inhibition of JNK (De Wever et al., 2004b). Finally, microarray data has been able to catalogue the genes expressed as a result of TGF-β-mediated myofibroblast differentiation over the time-course associated with this process (Chambers et al., 2003).

Together the epithelium, stromal fibroblast and myofibroblast signaling contributes significantly to the regulation of tumorigenesis. Although cell autonomous signaling should not be neglected, the tumor microenvironment consists of a complicated network of signals derived from many cell types. In addition to stromal-epithelial signaling the tumor microenvironment includes many transient cell populations including immune and inflammatory mediators that provide additional cytokines and growth factors upon TGF-β stimulation. The diverse nature of the cell populations responding to TGF-β in this complex environment necessitates careful exploration and cautious interpretation.

Within the analysis of TGF-β mediated signaling in the tumor microenvironment, all cell types should be considered to avoid missing vital regulatory interactions since most cell types respond to TGF-β signaling. With these central factors in mind, we continue to move toward effective manipulation of TGF-β pathway components *in vivo*, to improve individual and overall patient prognoses in cancer.

## Effects of TGF-β on immune mediators known to be associated with the tumor microenvironment

TGF-β signaling has been studied in the immune system with an intense focus on its contribution to cancer. In fact, TGF-β2 was first named glioblastoma-derived T cell suppressor factor (G-TsF) before being renamed, based on structural features that identified it as a TGF-β (Fontana et al., 1991). Many studies, including those involving ablation of the TGF-β genes in mice, illustrate a central role for TGF-β signaling in immune regulation. *Tgfb1*-/- mice show mis-regulation of the immune system resulting in a massive infiltration of immune cells to specific internal organs. The immune activity in this mouse model resembles an autoimmune disorder, graft rejection or viral infection (Kulkarni et al., 1993).

Immune evasion by tumor-derived TGF-β secretion has effects in both the innate and adaptive immune responses. The main cell-mediated immune components regulated by TGF-β in the tumor microenvironment include T lymphocytes, NK cells, cytotoxic T lymphocytes (CTLs), neutrophils (polymorphonuclear cells; PMNs) and macrophages. It is important to make the distinction between immune evasion and inflammation in the tumor microenvironment, since the two are intimately related. Inhibition of immune

effector functions (immune evasion) can contribute to the enhanced inflammation that can be associated with increased expression of cytokines, chemokines and growth factors in the tumor microenvironment.

Immune cell responses to TGF-β have been shown to regulate tumorigenesis. Expression of a dominant negative TβRII in T cells causes an increase in the immune response mediated by the CD8+ T cell population leading to ablation of grafted tumor cells *in vivo* (Gorelik and Flavell, 2001). Bone marrow cells engineered to carry a dominant negative TβRII can also contribute to an increase in survival of the host, when challenged with injection of tumor cells (Shah et al., 2002). In addition, experiments in which an antisense TGF-β1 or TGF-β2 RNA was expressed in tumor cells prior to injection into the host, demonstrate an increase in rejection of the resulting tumors. Rejection of tumor tissue in the presence of antisense TGF-β1 or TGF-β2 RNA was attributed to an increase in tumor cell immunogenicity due to diminished TGF-β signaling (Fakhrai et al., 1996; Park et al., 1997). In addition, administration of SD-208, a small molecule inhibitor of TGF-β signaling, also resulted in increased peripheral blood lymphocyte or purified T cell lytic activity and increased the secretion of interferon-γ and tumor necrosis factor alpha in response to mouse and human glioma cells (Uhl et al., 2004). These results, similar to earlier findings related to natural killer (NK) cell activation (Arteaga et al., 1993c), demonstrate that systemic inhibition of TGF-β signaling to reduce tumor progression can involve modification of immune effectors *in vivo*.

TGF-β was identified as a potent inhibitory signal for the immune response mediated by CTLs (Chen et al., 2005; Thomas and Massague, 2005; Wallick et al., 1990).

Mechanistically, the regulation of CTLs by TGF-β involves inhibition of perforin, granzyme A, granzyme B, Fas ligand, and interferon-γ expression (Thomas and Massague, 2005). Among these, the expression of granzyme B and interferon-γ were induced by TGF-β through Smad-responsive elements in their respective promoter regions. In human pancreatic adenocarcinomas about 90% of the tumors express mucin 1 (MUC1), and this protein has been used as an immunotherapeutic target for CTL killing of tumor cells (Mukherjee et al., 2001). TGF-β downregulates the MUC-1 induced CTL activity and decreases the expression of major histocompatibility complex (MHC) class I genes, which would normally contribute to an immune response elicited by the tumor. Inhibition of TGF-β signaling using a neutralizing antibody did not result in complete tumor regression, suggesting that other factors, such as upregulation of interleukin-10 or interleukin-4 expression in the tumor microenvironment, also contribute to the immune evasion *in vivo* (Hsiao et al., 2004; Mukherjee et al., 2001). TGF-β has also been linked to suppression of MHC class II expression in addition to the decrease in MHC class I expression (Geiser et al., 1993; Gorelik and Flavell, 2002; Johns et al., 1992; Lee et al., 1997; Ma and Niederkorn, 1995). Furthermore, TGF-β can inhibit immune responses to tumors elicited by NK cells and CD4+CD25+ regulatory T cells (Arteaga et al., 1993c; Chen and Wahl, 2003; Witham et al., 2003). It is widely accepted that TGF-β is an immunosuppressive factor for tumor infiltrating lymphocytes (TILs) and lymphokine-activated killer (LAK) cells through suppression of their effector functions. Inhibition of TGF-β can restore both TIL and LAK cell efficacy (Hsiao et al., 2004). The TIL cell population in this study was composed of mostly non-B or T cells (~90%) and the LAK cell population included non-B and T cells (~66%), monocytes (~18%), T cells (13%)

and B cells (~2%). In addition to TGF-β mediated regulation of LAK effector functions, TGF-β and interleukin-6 secreted from some tumors can induce the chemotactic behavior of this cell population, when purified and cultured *in vitro* (Delens et al., 1994). The negative effect of TGF-β on cytolytic activities mediated by LAK and CTL cells has long been known (Mule et al., 1988). LAK cells can be isolated from interleukin-2-activated NK cells, and interleukin-2 production that is often associated with NK cell activation is downregulated by TGF-β in T cells (D'Angeac et al., 1991). The downregulation of interleukin-2 prevents further activation of NK cells in the tumor microenvironment. The antigen-dependent activation of T helper cells can also be prevented by TGF-β (Gorelik and Flavell, 2002). In culture, one of the first direct demonstrations for this response came from several T helper cell lines that were resistant to TGF-β1. In the resistant cell lines, it was shown that the inhibition of T cell activation was due to a decrease in TGF-β receptor expression (Siepl et al., 1991). Further, the TGF-β pathway signaling has been shown to regulate T cell activation without physically interfering with interleukin-2 ligand binding or internalization (Ortaldo et al., 1991). These data suggest that, similar to results obtained in NK cells, the effect of TGF-β in the tumor microenvironment is T cell autonomous.

The mechanisms through which tumors evade the immune system include suppression of responses from neutrophils (polymorphonuclear cells; PMNs) that have the ability to eliminate Fas ligand (CD95L) expressing tumor cells (Hahne et al., 1996). Tumors that express the Fas ligand are able to eliminate immune mediators such as tumor reactive T lymphocytes that express the Fas receptor (CD95). In one set of experiments, injected malignant melanoma cells that express CD95L grew rapidly and formed tumors

in nude mice. In mutant mice deficient for expression of CD95, however, injection of the tumor cells resulted in a delay of tumorigenesis. This was due to the inability of the CD95L+ cells to initiate cell death of CD95+ cells including T lymphocytes (Hahne et al., 1996). The contribution of TGF-β to CD95L signaling is that it prevents neutrophils from killing cells that express CD95L. This inhibition of neutrophil activity was blocked using a soluble TGF-β receptor fusion protein and stimulated using TGF-β *in vitro*. *In vivo*, ectopic expression of TGF-β at the site of tumorigenesis protected the lesion from neutrophil-mediated tumor cell death. TGF-β signaling in the neutrophil prevents p38 MAP kinase activation in response to CD95 stimulation, and this has been suggested as a major factor in the inactivation of this important immune component (Chen et al., 1998). These surprising results regarding TGF-β reveal a unique way in which TGF-β helps to both evade the innate immune system, and promote the CD95L-mediated death of cells sensitive to this pathway including tumor reactive T lymphocytes.

The involvement of TGF-β in the evasion of cancers from the host immune response is also intimately related to inflammatory responses in the tumor microenvironment (Figure 2). TGF-β attracts immune and inflammatory mediators, while inactivating their ability to kill tumor cells. TGF-β attracts many cell types that contribute to inflammation including T cells, neutrophils, monocytes, NK cells, and mast cells (Adams et al., 1991; Gruber et al., 1994; Hanazawa et al., 1991; Maghazachi and al-Aoukaty, 1993; Reibman et al., 1991).

**Figure 2**. **Immune evasion and inflammation are directly regulated by TGF-β in the tumor microenvironment.** TGF-β is a chemoattractant for T cells, neutrophils, mast cells, macrophages, monocytes, natural killer and lymphokine-activated killer cells. In addition to acting as a chemoattractant, TGF-β can prevent the active and passive immune response within the tumor microenvironment. Evasion of the primary immune responses can lead to secondary immune evasion by allowing, for example, carcinoma cells to express CD95L that subsequently promotes T cell ablation. Carcinoma cells can express markers that would allow immune recognition and destruction in the absence of TGF-β. In carcinoma cells, TGF-β can change the level of MHC class I and II expression levels, and this would normally flag these cells for destruction. These cells are not eliminated due to the TGF-β-mediated immune suppression that prevents this response. The immune evasion mediated by TGF-β also contributes to enhanced inflammation. Classic inflammatory mediators and newly recruited inactive immune components synergistically contribute to the inflammation often observed within the tumor microenvironment.

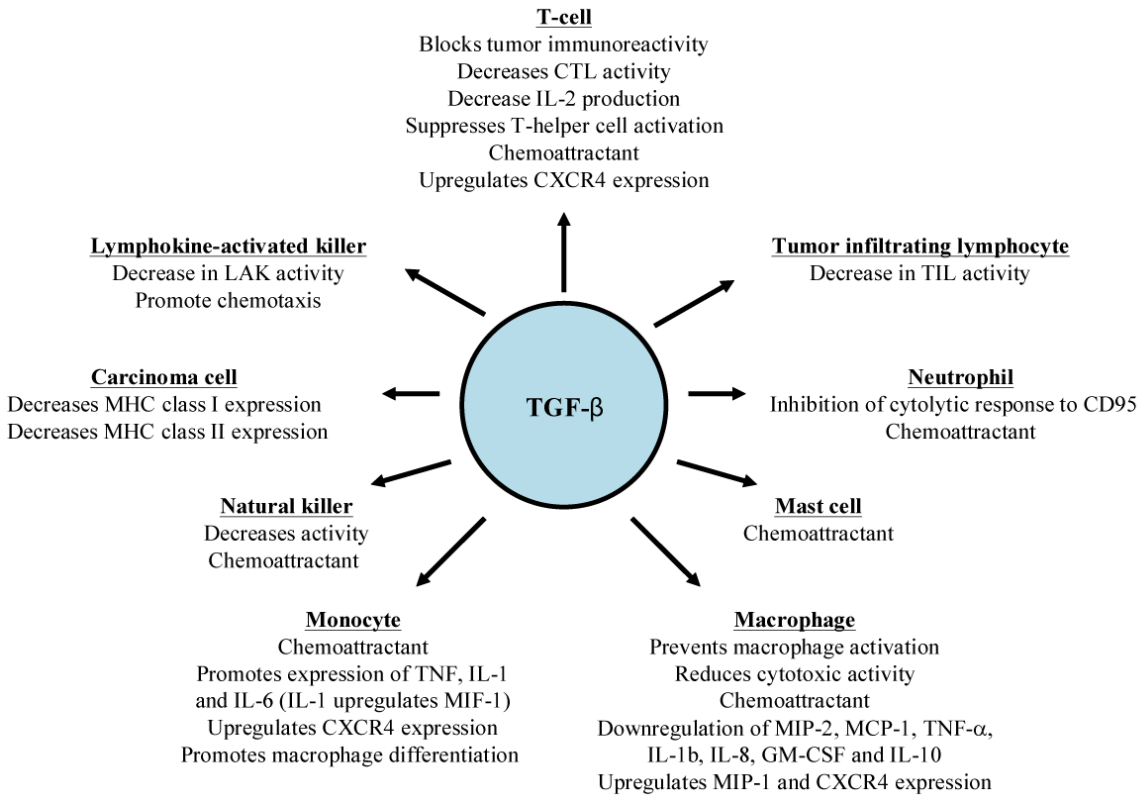


**Figure 2. Direct regulation of immune evasion and inflammation by TGF-β.**

TGF-β is a chemotactic factor for monocytes and promotes their expression of proinflammatory factors including tumor necrosis factor (TNF), interleukin-1 and interleukin-6 (Bogdan and Nathan, 1993; Fontana et al., 1992). Interleukin-1 has been shown to upregulate the expression of factors that promote cancer progression, such as macrophage migration inhibitory factor-1 (MIF-1), in the tumor microenvironment (Hira et al., 2005; Ren et al., 2004; Sun et al., 2005; Takahashi et al., 1998). TGF-β also upregulates the expression of the chemokine receptor CXCR4 on monocytes, macrophages and T cells, which contributes to their response to stromal cell-derived factor-1 (SDF-1/Cxcl12) (Buckley et al., 2000; Wang et al., 2001). The CXCR4-mediated response to SDF-1 may include transactivation of the epidermal growth factor (EGF) receptor, and increased motility and metastatic progression (Porcile et al., 2004; Sun et al., 2002).

In addition to acting as a chemotactic and cytokine stimulating factor, TGF-β promotes monocyte to macrophage differentiation (Bombara and Ignotz, 1992; Fontana et al., 1992). Similar to monocytes in the tumor microenvironment, tumor-associated macrophages (TAMs) are attracted to TGF-β and often secrete cytokines and growth factors that can further contribute to neoplastic progression (Coussens and Werb, 2002; Schoppmann et al., 2002; Young et al., 1996). *In vitro*, stimulation of LPS activated macrophages with TGF-β can result in downregulation of chemokines, macrophage inflammatory protein-2 (MIP-2), TNF-α, interleukin-1β, interleukin-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and to a lesser extent interleukin-10. In addition, TGF-β can stimulate the expression of the cytokine MIP-1.

The differences between the level of expression for these chemokines may be due to the relative dependence on NF-kB *versus* AP-1 for transcriptional regulation. Many chemokines such as TNF-α and MIP-2, are dependent on NF-kB while others, exemplified by MIP-1 are dependent on AP-1. TGF-β has been shown to stimulate AP-1 dependent transcription of MIP-1 in LPS-activated macrophages while inhibiting transcription of several NF-kB dependent chemokines. Further, the mechanism for stimulation of the AP-1 activity in the MIP-1 promoter depends on activation of Smad3 (Fadok et al., 1998; McDonald et al., 1999; Xiao et al., 2002). The chemokine monocyte chemoattractant protein-1 (MCP-1), is also expressed by macrophages, and TGF-β stimulates its expression, as opposed to the TGF-β mediated suppression of MCP-1 expression in fibroblasts (Kitamura, 1997). The expression of MCP-1 also depends on AP-1, but, unlike MIP-1, Smad3 activation inhibits its transcription. In Smad3-deficient macrophages, stimulation with TGF-β does not result in upregulation of MCP-1 transcription. Conversely, expression of a constitutively active Smad3 inhibits MCP-1 expression (Feinberg et al., 2004). These and other results suggest that monocytes and macrophages are initially attracted to TGF-β, and that monocytes are stimulated to terminally differentiate into macrophages yet are subsequently prevented from producing inflammatory proteins if the macrophages should become activated in the tumor microenvironment.

Activated macrophages offer another immune response to tumor cells that can be modified by TGF-β in the tumor microenvironment. Early observations suggested that the effect of TGF-β on macrophages in the tumor microenvironment was related to inhibition of macrophage activation (Haak-Frendscho et al., 1990). In the presence of

TGF-β, a human macrophage cell line was unable to be activated by interferon-γ or bacterial lipopolysaccharide (LPS). The lack of activation due to TGF-β stimulation prevented the macrophages from killing tumor cells *in vitro*. It was determined that TGF-β had no effect on the cytostatic activity of the macrophages, but the cytotoxic activity of the macrophages was greatly reduced at concentrations up five times as high as used for inhibition of macrophage activation (Haak-Frendscho et al., 1990). Furthermore, tumor-derived TGF-β induces immune suppressive activities from macrophages that may prevent T cell proliferation and contribute to additional tumor cell survival (Alleva et al., 1995).

## Regulation of tumor associated angiogenesis by TGF-β

Vasculogenesis occurs during development throughout embryonic life, and complemented and replaced by angiogenesis later during development, and in tumor progression and wound healing (Hanahan and Folkman, 1996). Over the years many groups have studied various aspects of the role of TGF-β signaling in the molecular mechanisms that regulate angiogenesis in the tumor microenvironment (Figure 3). The most informative mechanistic studies on the role of TGF-β have been performed *in vitro*, and little has been definitively shown *in vivo*. The problem with many correlative studies *in vivo*, and especially those using a systemic inhibition strategy, is that many different cell types in the tumor microenvironment are affected by the inhibition of TGF-β.

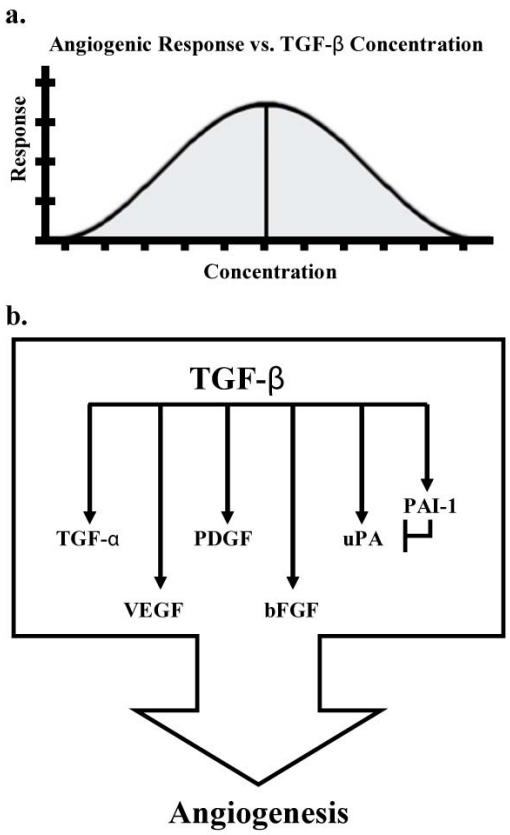


**Figure 3. TGF-β regulates angiogenesis in the tumor microenvironment.** TGF-β upregulates TGF-α, VEGF, PDGF, FGF-2 (bFGF), uPA and PAI-1 that all contribute to angiogenesis. uPA allows the release of active FGF-2, TGF-β and VEGF from the ECM. In addition to releasing the pro-angiogenic factors, uPA also produces plasmin from plasminogen and this has an anti-angiogenic effect. The upregulation of PAI-1 inhibits uPA and this has a pro-angiogenic effect. Together, the net effect of stimulation by TGF-β yields the release and activation of a pro-angiogenic gradient toward the tumor microenvironment.

Although studies *in vivo* may show a decrease in tumor endothelial cell density, but this may coincide with a change in tumor volume, epithelial cell growth and survival, immune response, inflammation or stromal-epithelial interactions. To address the endothelial cell response to TGF-β *in vivo*, specific ablation of TGF-β signaling in tumor endothelial cells would be informative. In culture, however, the current data are more convincing with respect to a role for TGF-β in angiogenesis.

*In vitro*, endothelial cells form capillary-like structures when embedded in type I collagen gels. This process *in vitro* requires either serum in the culture medium or addition of TGF-β to propagate angiogenesis. The effect of TGF-β in the culture medium was found to promote endothelial cell survival and induce formation of the capillary-like tubular structures. In this system, TGF-β induces the expression of TGF-α, which activates the EGF tyrosine kinase receptor on the same cells. Cells that are incubated with a soluble EGF receptor ectodomain or EGF receptor endogenous tyrosine kinase inhibitor AG1478 to block TGF-α signaling through the endogenous EGF receptor demonstrate that autocrine TGF-α signaling is required for TGF-β-induced capillary tube formation *in vitro*. Furthermore, this mechanism is dependent on EGF receptor-mediated activation of PI3 kinase and Akt and of p42/p44 Erk MAP kinase (Vinals and Pouyssegur, 2001). The endothelial cell response to TGF-β may depend on the level of stimulation for positive and negative regulation of angiogenesis, as demonstrated by early work in cell culture. One of the first examples for biphasic regulation of angiogenesis by TGF-β demonstrates that high levels of TGF-β inhibit, while low levels promote capillary tube formation *in vitro* (Pepper et al., 1993).

A central signaling pathway in the regulation of angiogenesis by TGF-β involves the expression of vascular endothelial growth factor (VEGF), which is currently considered as the most potent known angiogenic polypeptide. Stimulation of endothelial cells with TGF-β1 can lead to a significant increase of VEGF expression (Benckert et al., 2003; Breier et al., 2002; Donovan et al., 1997; Kitamura et al., 2003; Nagineni et al., 2003). Metastatic carcinoma cells express more VEGF in response to TGF-β than their non-metastatic counterparts (Donovan et al., 1997). In addition to the induction of VEGF expression by TGF-β1 made by the tumor cells, other factors such as hypoxia, can synergistically enhance VEGF expression in the tumor microenvironment (Breier et al., 2002). The expression of VEGF in response to TGF-β may be induced by MAP kinase signaling and involve the AP-1 and HIF (hypoxia-inducible factor) transcription factors (Nagineni et al., 2003; Shih and Claffey, 2001; Tokuda et al., 2003).

The induction of angiogenesis in the tumor microenvironment may include the regulation of expression of other factors by TGF-β, including platelet-derived growth factor (PDGF) and fibroblast growth factor-2 (FGF-2, also known as basic FGF). In addition to VEGF and TGF-α, TGF-β induces the expression of PDGF and FGF-2 in many cell types that are commonly associated with the tumor microenvironment (Leof et al., 1986; Pepper, 1997). TGF-β and PDGF are both able to increase the expression of VEGF and FGF-2, whereas hypoxic conditions induce the expression of VEGF only *in vitro* (Brogi et al., 1994). In the tumor microenvironment, however, hypoxic regions in the center of a tumor are often associated with concurrent TGF-β expression. In addition to hypoxia, other indirect effects can further contribute to angiogenesis. For example, the

TGF-β-induced recruitment of inflammatory cells provides additional cytokine and growth factor signaling to the tumor microenvironment.

The expression of the angiogenic factor FGF-2 in response to TGF-β has been shown to contribute to TGF-β-activated endothelial cell organization. *In vitro*, FGF-2 alone can induce capillary tube-like structures from endothelial cells in a monolayer. However, the appearance and length of the capillary-like structures that are formed in response to both TGF-β and FGF-2 are more reminiscent of those formed *in vivo*. This convergent signaling to regulate capillary morphogenesis further parallels data *in vivo*, involving the observations of altered lumen size in the *Tgfb1*- or *Tgfbr2*-defective mice (Pepper, 1997). It is likely that, in the tumor microenvironment, inflammation, hypoxia, PDGF, FGF-2, VEGF and a number of other factors synergize with TGF-β to promote angiogenesis that contributes to tumor progression and metastasis.

Importantly, TAMs can produce urokinase-type plasminogen activator (uPA) in response to stimulation with TGF-β, and the expression of uPA leads to conversion of plasminogen to plasmin (Hildenbrand et al., 1998). Plasmin has been shown to mediate anti-angiogenic signaling through several mechanisms including fibrinolysis. In addition to its role in the production of anti-angiogenic signaling, uPA also induces the release of FGF-2, hepatocyte growth factor (HGF), TGF-β and VEGF from latency-associated complexes (Falcone et al., 1993; Mazar et al., 1999). However, TGF-β also induces the expression of plasminogen activator inhibitor (PAI)-1, which inhibits the activity of uPA. In the presence of PAI-1, the activity of uPA is inhibited and fibrin formation is increased. Furthermore, the combination of VEGF and fibrin has been associated with an increase in angiogenesis (Mazar et al., 1999).

## TGF-β based therapeutic strategies in cancer

In human carcinoma, tumor cell autonomous TGF-β expression is often increased while expression of the receptor dependent signaling components is decreased, mutated or silenced. It is now generally accepted that TGF-β is an early tumor suppressor that can subsequently promote progression through tumor cell autonomous and host-tumor interactions. During tumor progression, TGF-β signaling has been shown to promote metastasis, while the complete absence of TGF-β signaling in the carcinoma cell population has also been shown to increase the rate of metastasis. TGF-β also regulates important host-tumor interactions including immune evasion and stimulation of angiogenesis during tumor progression. In addition it has been shown that TGF-β signaling mediated by stromal fibroblasts can suppress tumor formation and progression in adjacent epithelia. It is clear that a large number of cell type and context dependent factors contribute to the dynamic regulatory roles for TGF-β in each unique tumor microenvironment.

The complexity associated with TGF-β dependent regulation of tumor progression (Figure 4, A-E) is further convoluted by the disease itself, since cancers cells often demonstrate mis-regulation of multiple networks that are further amplified to promote progression (Hanahan and Weinberg, 2000; Hill et al., 2005; Rajagopalan et al., 2003; Vogelstein and Kinzler, 2004).

**Figure 4. Visual representation of results from the current literature related to TGF-β signaling in cancer.** (A) TGF-β tumor suppressor activity. TGF-β signaling has a positive correlation with tumor suppression early during tumorigenesis, and as a primary tumor progresses this correlation decreases as it becomes a tumor promoter. (B) TGF-β tumor promoter activity. Initiation and early progression negatively correlate with the tumor promoter activity of TGF-β. As a tumor progresses, there is an increasing correlation with tumor promoting activity mediated by TGF-β signaling. (C) During tumor progression there is a positive correlation with the mutations acquired in TGF-β signaling components. (D) Systemic inhibition of TGF-β reduces the aggression during tumor progression. In many cases, systemic inhibition of TGF-β will result in a decrease of metastases and therefore negatively correlates with aggression as compared to untreated controls. (E) Epithelial cell autonomous ablation of TGF-β signaling results in a delayed initiation and progression. There are mixed results in the literature regarding the effect on advanced progression and metastasis (both positive and negative correlations have been reported). (F) Ablation of TGF-β signaling in fibroblasts can potentiate carcinoma formation in adjacent epithelial populations over time.

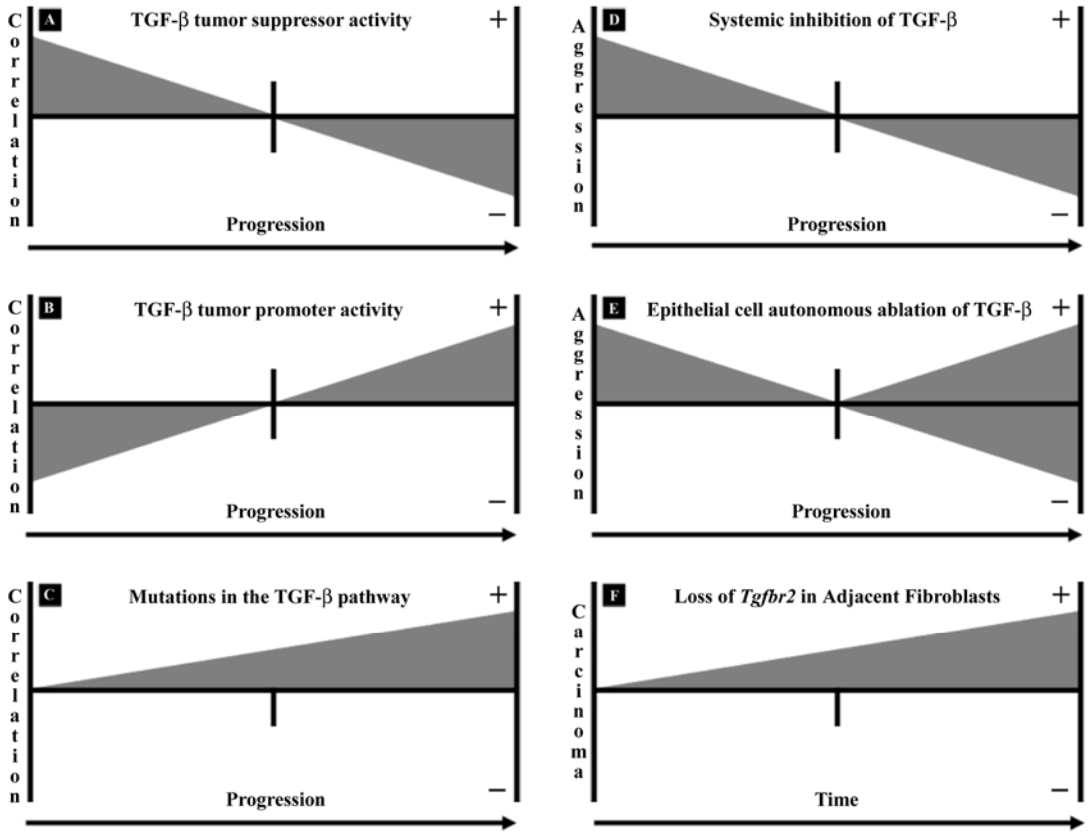


**Figure 4. Visual representation of results from the current literature related to TGF-β signaling in cancer.**

As a result of this inherent complexity, TGF-β based therapeutic strategies must be carefully considered prior to administration. In opposition to general clinical application of an anti-TGF-β therapy, recent emphasis has been placed on global molecular profile screening. The application of various profiling techniques may help to indicate when a specific TGF-β based treatment strategy should be administered, to maximize efficacy while reducing the adverse effects potentially associated with this type of intervention. However, with the advance of global profiling technologies and their concurrent application during the screening process, we are moving cautiously toward tailored therapies that include regulation of this potent regulatory pathway in cancer.

The complex nature of TGF-β signaling and crosstalk in the tumor microenvironment presents a unique challenge, as well as an opportunity for therapeutic intervention strategies targeting cancer. Currently, the TGF-β pathway has been targeted using strategies including modification of immune components or delivery of small molecule inhibitors and soluble protein or antisense compound inhibitors. Immunotherapeutic strategies, at the current time have only been used to target the TGF-β pathway in animals, however these approaches are appealing since a major contribution to tumor progression mediated by TGF-β is evasion of the immune system (Chen and Wahl, 2003; de Visser and Kast, 1999; Gorelik and Flavell, 2002; Wahl et al., 2004). The strategies employed in immunotherapy related to TGF-β usually decrease TGF-β signaling in an immune component, prior to reconstitution in a tumor bearing recipient, thereby permitting a productive interaction with cancer cells. Alternatively, systemic delivery of compounds used to inhibit TGF-β usually abrogate all host-tumor interactions regulated by TGF-β including those involving immune evasion, angiogenesis, stromal-

epithelial crosstalk and tumor cell autonomous signaling (Bierie and Moses, 2006a; Goumans et al., 2003a; Lebrin et al., 2005; Muraoka-Cook et al., 2005a). Due to the immune mediated disease and lethality associated with genetic ablation or inhibition of TGF-β signaling in mice (Gorelik and Flavell, 2000; Kulkarni et al., 1993; Leveen et al., 2002; Shull et al., 1992), it was unclear if inhibiting this pathway to treat cancer would be compatible with patient survival when delivered for a sustained duration *in vivo*. However, it has recently been shown that a lifetime exposure to systemic soluble TGF-β1/3 or pan-TGF-β inhibitors in mouse models did not result in significant adverse effects (Ruzek et al., 2003; Yang et al., 2002b). These studies have demonstrated that TGF-β specific inhibition should be compatible with long-term survival when administered to humans for a sustained duration *in vivo*. Importantly, the mechanism of action is known for many of the published inhibitors, and some of these agents are currently being evaluated in human clinical trials (Yingling et al., 2004).

It has been known for many years that one of the major contributions to tumor progression mediated by TGF-β is evasion of the immune response (de Visser and Kast, 1999; Fontana et al., 1991; Fontana et al., 1992; Gorelik and Flavell, 2002; Smyth et al., 2006). In addition to evasion of tumor cell immunoreactivity, TGF-β attracts immune components to the tumor microenvironment, thereby permitting the expression of additional tumor promoting factors (Figure 5). The first definitive results indicating that TGF-β mediated suppression of T-cell populations could be subverted to eliminate cancer came from experiments using transgenic mice that expressed a dnTβRII in all T-cells resulting in suppression of metastasis when challenged with B16-F10 or EL-4 cell lines (Gorelik and Flavell, 2001).

**Figure 5. Previously described roles for TGF-β in the tumor microenvironment and drugs designed to attenuate systemic signaling.** Transforming growth factor-beta (TGF-β) is a potent regulator of T-cell, neutrophil, monocyte, macrophage, natural-killer (NK)-cell, carcinoma-associated fibroblast and carcinoma-cell-autonomous signaling in the tumor microenvironment. In addition, TGF-β contributes to the regulation of angiogenesis through direct and indirect mechanisms. TGF-β stimulates the migration of fibroblasts, T cells, neutrophils and monocytes, and influences their behaviour to suppress or promote tumor progression. TGF-β inhibits T-cell function, the secretion of cytolytic factors from cytotoxic T cells and stimulates monocytes to differentiate into macrophages, but inhibits the effector function of macrophages when present. TGF-β might stimulate tumor-homing T cells and macrophages through the upregulation of CXCR4 in response to CXCL12 secretion by carcinoma-associated fibroblasts. TGF-β enables the expression of FAS ligand (FASL) by inhibiting the activation of neutrophils, cells that would normally kill FASL-expressing cells. This is an important role for TGF-β, as the expression of FASL promotes immune evasion by inducing cell death in FAS-expressing cells, including tumor-reactive T-cell populations. TGF-β signaling also results in a reduction of major histocompatibility complex (MHC) class I and II gene expression that would normally induce NK-cell activation, but TGF-β has been shown to inhibit NK-cell activation, which further contributes to the promotion of tumor progression. Finally, TGF-β signaling influences tumor-cell-autonomous signaling that can suppress or promote progression and metastasis depending on the context of stimulation. These interactions are often manipulated individually, through immunotherapy, or globally, through systemic inhibition strategies, in cancer research or clinical therapy. References are available in the corresponding text. GMCSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; IL, interleukin; MCP1, macrophage chemoattractant protein 1; MIP, macrophage inflammatory protein; MST1, macrophage stimulating 1; TNF, tumor-necrosis factor.

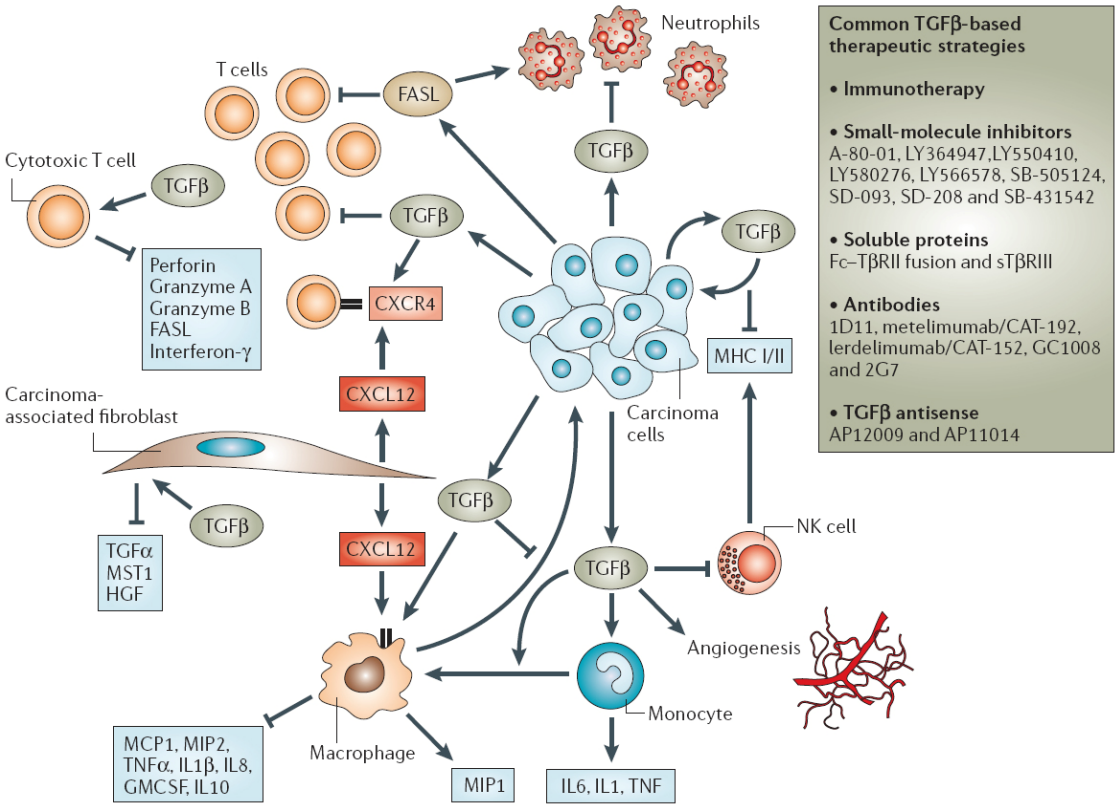


**Figure 5. Previously described roles for TGF-β in the tumor microenvironment and drugs designed to attenuate systemic signaling.**

Another exciting approach that has recently been used to eliminate cancer in mice, based on the original work describing subversion of immune evasion to eliminate tumor cells, is the adoptive transfer of tumor-reactive, TGF-β insensitive, CD8+ T-cells using a mouse prostate cancer model (Zhang et al., 2005). In this system, donor mice were primed with irradiated TRAMP-C2 prostate cancer cells and then $CD8^+$ T-cells isolated from the spleen of these mice were expanded *ex vivo*. A dominant negative TGF-β type II receptor was then introduced into the CD8+ T-cells through retroviral infection to render these cells insensitive to TGF-β. *In vitro*, these engineered cells demonstrated specific cytotoxicity for the TRAMP-C2 cell line, and after adoptive transfer of these cells into mice a marked reduction or complete elimination of pulmonary metastases was observed. This study also showed that only the engineered TGF-β insensitive T-cells were able to penetrate the tumor in order to mediate apoptosis, and the engineered cell numbers declined in tumor free animals over time, however they persisted in tumor bearing mice (Zhang et al., 2005). These results indicate that the high levels of TGF-β secreted by the TRAMP-C2 cell line (as demonstrated by ELISA) prevented endogenous T-cell cytotoxicity that would otherwise eliminate tumor cells *in vivo*. Together these studies demonstrate a general principle — that TGF-β produced by tumor cells can suppress a functional immune response, and inhibition of this suppressive signal enhances recognition and destruction of tumor cells *in vivo*.

The TGF-β pathway has recently been targeted through the use intracellular strategies involving administration of small molecule inhibitors of the TβRI and TβRII kinase domains. Several well characterized specific small molecule inhibitors have been identified for this purpose, including A-80-01, LY364947, LY550410, LY580276,

LY566578, SB-505124, SD-093, SD-208 and SB-431542 (DaCosta Byfield et al., 2004; Ge et al., 2004; Inman et al., 2002; Peng et al., 2005; Tojo et al., 2005; Uhl et al., 2004; Yingling et al., 2004). The Alk-5 small molecule inhibitors can also target the TβRI Alk-4 and Alk-7 receptors, and therefore results obtained through their application may not directly correlate with TGF-β specific signaling and will require substantial validation (Peng et al., 2005). Inhibition of Alk-4 and Alk-7, in addition to TGF-β specific Alk-5 may have an influence on tumorigenesis. In particular Alk-4 may be important in the regulation of tumorigenesis, since upregulation of Alk-4 expression in the MMTV-Neu mouse model has been demonstrated in correlation with activated Smad2 and loss of Alk-5 expression (Landis et al., 2005). In addition, expression of activin A, the ligand responsible for Alk-4 activation, has been associated with esophageal carcinoma, providing a clinical link to activation to this pathway in human cancer (Yoshinaga et al., 2003). The use of specific small molecule inhibitors, as highlighted through studies involving the SD-208 and SD-093 compounds, can result in the potent regulation of cancer cells *in vitro* and *in vivo*. SD-208 could regulate growth of intracranial SMA-560 gliomas in syngeneic mice, resulting in prolonged survival that correlated with increased immune infiltration without significant changes in proliferation, apoptosis or angiogenesis (Uhl et al., 2004). The positive effect on tumor reactive immune regulation mediated by the SD-208 compound seems to be direct as demonstrated through parallel analyses conducted *in vitro* (Uhl et al., 2004). In another study, the SD-093 inhibitor was shown to negatively regulate invasiveness without changing the morphology or growth rate in SMAD4 deficient pancreatic carcinoma cells (Subramanian et al., 2004). *SMAD4* expression is often decreased or lost in cancer, and this suggests that the use of small

molecule inhibitors in this context has potential for therapeutic application. These results, in addition to a growing number of similar small molecule inhibitor studies, have demonstrated efficacy in blocking this pathway through attenuation of intracellular kinase signaling to modify tumor cell behavior *in vitro* and *in vivo*.

In contrast to the current small molecule inhibitors, soluble proteins including Fc:TβRII (Cosgrove et al., 2000), the soluble type III TGF-β extracellular domain (Bandyopadhyay et al., 1999) and TGF-β specific antibodies are highly specific for attenuation of extracellular TGF-β mediated pathway activation. The Fc:TβRII fusion protein is thought to bind TGF-β1 and TGF-β3 with high affinity, while the soluble TβRIII extracellular domain (sTβRIII) is able to bind all three TGF-β isoforms. The expression of the sTβRIII protein, similar to the results obtained through administration of the Fc:TβRII fusion protein, resulted in reduced tumor incidence, growth rate and lung metastases when expressed in MDA-231 cells inoculated into nude mice (Bandyopadhyay et al., 1999; Muraoka et al., 2002). It was later shown that administration of a recombinant sTβRIII protein significantly inhibited tumor growth and metastases to the lung (Bandyopadhyay et al., 2002). The use of the relatively new Fc:TβRII and sTβRIII inhibitors *in vitro* and *in vivo*, have produced results similar to those obtained through the use of TGF-β neutralizing antibodies over the past decade. The most prominent antibodies that have been used for the purpose of systemic inhibition include 1D11 (Dasch et al., 1989), metelimumab/CAT-192 (clinicaltrials.gov ID: NCT00043706), lerdelimumab/CAT-152 (Cordeiro et al., 1999; Thompson et al., 1999), GC1008 (clinicaltrials.gov ID: NCT00125385) and 2G7 (Lucas et al., 1990). In experimental cancer research, the neutralizing 2G7 pan-TGF-β mouse monoclonal IgG2B

antibody has been particularly useful in the identification and verification of unique regulatory roles for TGF-β signaling in the tumor microenvironment. One of the early applications of this antibody in the study of cancer, demonstrated that TGF-β can overcome estrogen receptor dependence to promote tumorigenesis, as shown in experiments using modified estrogen receptor positive (ER+) MCF-7 cells in ovariectomized or castrated nude mice (Arteaga et al., 1993a). Normally the MCF-7 cells will not form tumors when grafted into ovariectomized nude mice without estrogen supplementation. When a TGF-β1 expression plasmid was introduced into this cell line, tumors developed in 100% of the mice, and these tumors could be completely ablated using 2G7 (Arteaga et al., 1993a). In a complementary approach, estrogen independent MDA-231 cells were used to address the effect of TGF-β on tumorigenesis (Arteaga et al., 1993b). When MDA-231 cells were grafted into nude mice they were able to form metastatic tumors. Tumorigenesis and metastasis in this model could be blocked using the 2G7 antibody. Interestingly, the MDA-231 cells inhibited, and 2G7 enhanced, spleen NK-cell activity. Further, conditioned medium from MDA-231 cells inhibited lymphocyte-mediated NK activity *in vitro*, and this correlated with results demonstrating that the 2G7 neutralizing antibody was unable to inhibit MDA-231 tumors in NK cell deficient hosts (Arteaga et al., 1993b; Arteaga et al., 1993c). In the years following these initial systemic extracellular TGF-β based inhibition studies, many subsequent reports have further illustrated the importance of cell autonomous and host-tumor interactions such as immune evasion, in the regulation of cancer mediated by TGF-β in the tumor microenvironment.

Early antisense therapy used to treat intracranial rat glioma, based on inhibition of *Tgfβ2* demonstrated some success, but more importantly demonstrated that TGF-β2 in this context would be a good target for antisense based intervention (Fakhrai et al., 1996; Liau et al., 1998). A new type of therapeutic agent composed of soluble phosphorothioate oligodeoxynucleotide (S-ODN) antisense specific for human *TGFβ2* mRNA has recently been used to target the TGF-β pathway *in vivo* (Schlingensiepen et al., 2005a; Schlingensiepen et al., 2005b). Although limited information is available regarding the use of this compound in treating human cancer, it is currently in clinical trials, and some interesting information has been reported regarding delivery of this compound to human cancer patients *in vivo*. The compound (AP 12009) was initially tested for use in treating brain tumors, and it was shown to be well tolerated when delivered into the brain parenchyma. The sequence used for AP 12009 was selected based on its ability to mediate TGF-β2 silencing in cells without the use of a carrier such as lipofectin. The AP 12009 compound was also screened for efficacy *in vitro* using TGF-β2 expression, proliferation, migration and effect on immune suppression as markers for activity in glioma, pancreatic carcinoma and malignant melanoma cell lines. The AP 12009 compound is currently being evaluated in clinical trials as a potential glioma, pancreatic carcinoma and malignant melanoma therapy while a second compound targeting *TGFβ1* mRNA (AP 11014) is in preclinical development for the treatment of human non-small-cell lung, colorectal and prostate cancers (Schlingensiepen et al., 2005a; Yingling et al., 2004). Initial results from human clinical trials, involving treatment of high-grade glioma with AP 12009 administered locally into brain tumors via convection-enhanced delivery, have demonstrated that this strategy has the potential for

clinical application. In the initial phase I/II open-label dose escalation study, some of the first data indicating efficacy for this strategy was obtained. Specifically, the compound was able to significantly prolong the median time to relapse compared with the published relapse times for temozolomide chemotherapy. In addition, seven of twenty-four patients demonstrated stabilization of their disease, and two patients were shown to be in complete remission after treatment. The initial studies using AP 12009 show potential for therapeutic intervention without significant adverse effects, and this S-ODN compound has now moved to a phase IIb clinical study for further evaluation in high-grade glioma (Schlingensiepen et al., 2005a).

## Summary

TGF-β has a clear role in cancer cell autonomous regulation of tumorigenesis, but it also has several roles in the tumor microenvironment that contribute to the progression of disease. In many types of normal epithelium and during early tumorigenesis, TGF-β acts as a tumor suppressor while later in progression it is a tumor promoting factor (Akhurst and Derynck, 2001; Cui et al., 1996). The effects of TGF-β can be attributed to tumor cell autonomous, immune, inflammatory, angiogenic and stromal signaling *in vivo*. Due to this broad spectrum of activity, the balance of TGF-β signaling should be considered when designing a therapeutic targeting strategy that involves modification of this pathway *in vivo*. As an example, systemic TGF-β blockers may inhibit the fibroblast signaling, angiogenic stimulation and immune evasion while promoting epithelial cell proliferation and this could result in subsequent tumor progression. Conversely, proliferation of the epithelial cells may be increased by TGF-β stimulation and the net

result would then be tumor inhibition. It is clear that in order to develop effective treatments based on modification of TGF-β signaling, we must understand the role for this pathway in each cell type known to contribute to the tumor microenvironment.

Immune evasion has been associated with tumor progression, and TGF-β is a potent mediator of this process. Lack of TGF-β1 resulted in an overactive immune system and this clearly demonstrated a role for TGF-β in immune regulation *in vivo*. In cancer, several groups have shown that tumor progression can be decreased through ablation of TGF-β signaling specifically in the immune system. The effects of TGF-β signaling on immune invasion are both direct and indirect. The direct effects mediated by TGF-β include preventing the activation and function of immune killer cells. Indirect effects on immune evasion can be exemplified by neutrophil suppression, which allows sustained expression of CD95L in the tumor microenvironment. The increase in CD95L expression further prevents T lymphocyte-mediated tumor targeting. Immune evasion prevents destruction of tumor tissues, but it also contributes to inflammation. Many of the immune components that are suppressed by TGF-β are also attracted to TGF-β expressing tissues. The chemotactic activity of TGF-β can promote immune infiltration of the tumor tissues. The presence of infiltrating immune cells in the tumor microenvironment leads to expression of many cytokines and growth factors that can contribute to tumor progression. The current data suggests that together, TGF-β-mediated immune evasion and enhanced inflammation work synergistically to promote tumor progression.

In the tumor microenvironment, a large portion of the TGF-β literature has focused on the epithelial tumor cell autonomous and immune evasion responses in

cancer. There are however other key factors for TGF-β-mediated signaling in the areas of angiogenesis and stromal fibroblast contributions to disease initiation and progression. TGF-β has been found to promote the upregulation of angiogenic ligands in tumor cells and this recruits endothelial cells to the growing tumor. Hypoxia can further enhance the TGF-β-mediated angiogenic ligand production while concurrently upregulating TGF-β1 transcription. Upon recruitment to the tumor microenvironment, TGF-β helps to promote angiogenic vessel organization and integrity. To promote migration and growth of cells in a tumor there must be a matrix to build upon. Many of the matrix proteins are produced by stromal fibroblasts, but this is only one function for these cells in the tumor microenvironment. In addition to the production of collagen and other essential matrix proteins it is now clear that stromal fibroblasts produce potent signals that are able to modulate adjacent cell populations. In the case of epithelial cells, the fibroblasts juxtaposed within the tumor microenvironment can have a profound influence on tumor progression. Stromal fibroblasts may become activated in a tumor, and this produces permanent changes in gene expression that modify their respective tumor stromal-epithelial interactions. Current data suggests that lack of a TGF-β signal in the stromal fibroblasts, through ablation of TβRII in the fibroblasts for example, can cause production of factors that subsequently act upon adjacent cancer cells to promote tumorigenesis.

The tumor microenvironment is complex, and we are only now beginning to understand the enormous scope of interactions that result from the presence of TGF-β signaling in this context. The current data relating to TGF-β spans two decades, and it is likely that the next two decades will provide many more key mechanisms without

exhausting the need for continued persistent study of this central signaling pathway in cancer. Our current results presented herein, explore the role for TGF-β signaling in the regulation of mammary development and tumorigenesis. The data that we have produced strongly suggest that TGF-β can suppress mammary epithelial cell differentiation, tumorigenesis and metastasis *in vivo*. We have demonstrated that TGF-β signaling in mammary carcinoma cells can promote apoptosis and mediate interactions with the adjacent fibrovascular stroma. We have also shown that TGF-β signaling regulates inflammatory gene expression and recruitment of bone marrow derived cells that are known to promote metastasis. In addition, we have now demonstrated that TGF-β signaling can regulate tumor cell heterogeneity in the tumor microenvironment. At the molecular level, we have identified and validated gene expression signatures that represent carcinoma cell specific loss or activation of TGF-β signaling. The carcinoma cell gene expression signatures further substantiated a role for TGF-β in regulation of chemokine expression and were able to provide a link to progression of human disease. Importantly, the gene expression signature associated with loss of TGF-β signaling in mammary carcinoma cells significantly correlated with an increased risk of relapse in human Luminal A, ER+ or lymph node positive breast cancer. Together, our results and the previously published literature strongly suggest, that loss the carcinoma cell response to TGF-β signaling during progression of disease, can significantly increase the risk of relapse and poor outcome for breast cancer patients.

CHAPTER II

# TGF-β DEPENDENT REGULATION OF NORMAL MAMMARY DEVELOPMENT

## Introduction

The TGF-β pathway is known to significantly regulate mammary development and tumorigenesis. During mammary development, the TGF-β isoforms TGF-β1, TGF-β2 and TGF-β3 are expressed in distinct spatial and temporal patterns (Robinson et al., 1993; Robinson et al., 1991; Streuli et al., 1993). It has been shown that all three isoforms are present during virgin mammary development and pregnancy. TGF-β1 and TGF-β3 levels were shown to be more abundant than TGF-β2 during virgin development and associated predominantly with the terminal end bud and ductal structures. However, TGF-β1 and TGF-β3 were also expressed in the mammary fat pad in the absence of epithelium. During pregnancy all three isoforms were abundantly expressed in mammary alveoli, ducts and fat pad. Upon parturition, all three TGF-β isoforms were significantly downregulated and only TGF-β3 was detected at low levels in mammary alveoli (Robinson et al., 1991). During involution, TGF-β3 expression was markedly increased through a process regulated by milk stasis and has been shown to be more abundant than TGF-β1 or TGF-β2 (Clarkson et al., 2004; Nguyen and Pollard, 2000; Stein et al., 2004; Streuli et al., 1993).

Relevance for TGF-β signaling during mammary development was shown in early studies using ethylene vinyl acetate copolymer (EVAc) pellets containing TGF-β that were implanted in the mammary fat pad of five week old virgin mice (Silberstein and Daniel, 1987). The TGF-β implants were able to inhibit growth terminal end buds (TEBs) into the fat pad, thereby preventing ductal elongation. It was further shown that the effect of growth inhibition by TGF-β was reversible. The effect of TGF-β signaling was specific to the epithelium and had no observed effect on the proliferation of adjacent stroma (Daniel et al., 1989).

It was later shown that TGF-β1 +/- mammary epithelium, which produced approximately 10% of the TGF-β1 present in wild type tissue, exhibited aberrant mammary lobuloalveolar proliferation *in vivo* (Ewan et al., 2002; Geiser et al., 1993; Kulkarni et al., 1993). Further, TGF-β could restrain hormone dependent mammary epithelial cell proliferation (Ewan et al., 2002). These results correlated well with data obtained through expression of a dominant negative type II TGF-β receptor mutant under control of the MMTV promoter/enhancer (MMTV-DNIIR) (Gorska et al., 1998). Attenuation of TGF-β signaling using this strategy resulted in mammary alveolar hyperplasia and precocious terminal differentiation (Gorska et al., 1998). Further, at twenty weeks of age the hyperplasia positively correlated with diestrus and estrus stages of development (Gorska et al., 1998). During pregnancy, the MMTV-DNIIR mice also exhibited an increased rate of precocious differentiation (Gorska et al., 2003), further linking the TGF-β pathway to regulation of the mammary epithelial cell response to endocrine signaling *in vivo*.

TGF-β3 signaling has been associated with regulation of the first stage of mammary involution (Nguyen and Pollard, 2000). To determine the effect of TGF-β3 signaling during mammary development an initial approach involved the use of transplanted glands derived from TGF-β3 null mice that have been shown to die shortly after birth (Nguyen and Pollard, 2000; Proetzel et al., 1995). The transplants indicated that the apoptotic effect involved autocrine signaling during mammary involution. Further, transgenic overexpression of TGF-β3 under control of the *Blg* promoter resulted in increased signal transducer and activator of transcription 3 (Stat3) phosphorylation and positively correlated with increased cell death in the first three days of post-partum transgene expression (Nguyen and Pollard, 2000).

Subsequently, the MMTV-DNIIR mouse model was used to demonstrate a similar delay in mammary involution upon forced weaning of pups during lactation (Gorska et al., 2003). In addition mammary epithelial cell specific loss of Smad3, a well characterized downstream mediator of TGF-β signaling, was shown to have a similar effect on apoptotic induction during the first three days of involution (Yang et al., 2002c). Interestingly deletion of Smad4, a central mediator and Smad3 binding partner that regulates canonical TGF-β signaling, in mammary epithelium did not reveal a corresponding involution defect (Li et al., 2003).

TGF-β and Prolactin (Prl) exert opposing effects on mammary epithelial cell survival (Bailey et al., 2004). It has been shown that in the non-transformed HC11 murine mammary epithelial cell line, TGF-β1 inhibits Akt phosphorylation at S473. Conversely, Prl activates Akt S473 phosphorylation and is dominant over the effect of TGF-β in the activation of this pathway. It was shown that TGF-β mediated apoptosis in

this model system was dependent upon downregulation of Akt signaling. Conversely, Prl promoted cell survival through upregulation of Akt signaling. It was further shown, that the hyperplastic growth of mammary epithelium observed in MMTV-DNIIR mice was dependent upon Prl signaling *in vivo* (Bailey et al., 2004). These results were correlated with reduced apoptosis in the MMTV-DNIIR mouse model after nipple sealing experiments designed to induce localized involution of mammary tissue *in vivo* (Bailey et al., 2004).

The literature has suggested that TGF-β signaling could regulate initiation of early mammary involution (Bailey et al., 2004; Gorska et al., 2003; Nguyen and Pollard, 2000; Proetzel et al., 1995; Yang et al., 2002c). Several reports have also suggested that TGF-β signaling was able to suppress terminal differentiation of mammary epithelium (Cocolakis et al., 2008; Gorska et al., 2003; Gorska et al., 1998). However, it was unclear what impact either effect had on the global process of mammary involution. Previously, we have used an MMTV-Cre transgene to delete *exon 2* from the *Tgfbr2* gene in mammary epithelium, however we observed a gradual loss of TβRII deficient epithelial cells that precluded an accurate study of the role for TGF-β signaling during involution timepoints (Chytil et al., 2002; Forrester et al., 2005). To address this issue, using the WAP-Cre transgene and our floxed TβRII mouse model (Chytil et al., 2002; Wagner et al., 1997), we have now selectively ablated TβRII signaling in mammary epithelium predominantly associated with late pregnancy, lactation and early involution timepoints. Using this strategy, we have been able to determine that many of the processes that should occur during the first-to-second phase transition of mammary involution are not significantly altered in the absence of TGF-β signaling. Notably, we

were able to detect the silencing of Na–Pi type IIb co-transporter (Npt2b) expression by day three of involution in both control and TβRII deleted tissues. In addition, we did not detect a significant difference in the level of apoptosis or metalloproteinase induction at day three of involution. However, by day seven of involution Npt2b, a previously described Jak/Stat dependent terminal differentiation marker (Long et al., 2003; Miyoshi et al., 2001; Shillingford et al., 2003), was re-initiated in TβRII deficient tissues. Together, the results suggest that induction of the second irreversible stage of mammary involution is not significantly altered when TGF-β signaling is completely abrogated. However, late during the second stage of mammary involution, suppression of terminal differentiation by TGF-β is required to efficiently remodel the mammary tissue *in vivo*.

## Experimental Procedures

### Animal models

WAP-Cre, TβRII$^{(WKO)}$Rosa26R, TβRII$^{(MKO)}$ and MMTV-DNIIR mice were bred and genotyped as previously described (Bierie et al., 2008; Forrester et al., 2005; Gorska et al., 2003; Gorska et al., 1998; Soriano, 1999; Wagner et al., 1997). Virgin timepoints for the TβRII$^{(WKO)}$Rosa26R and WAP-Cre control analyses included twelve and twenty weeks of age. Four to six mice were used for each genotype at the virgin timepoints. Virgin tissues from the TβRII$^{(MKO)}$ model were collected at nineteen weeks of age and the virgin MMTV-DNIIR tissues were collected at twenty weeks of age. Mice were checked for plugs after breeding to determine the first day of pregnancy for the day fifteen

pregnancy timepoint. Four mice were used for each genotype at day fifteen of pregnancy. Day three of lactation was used for the corresponding lactation timepoint with five mice in the control group and four mice in the TβRII$^{(WKO)}$Rosa26R group. Forced involution was conducted by allowing pups to suckle for ten days after parturition, then removing the pups to induce involution. A range of five to eight mice were collected for each genotype and timepoint at day one and two after forced involution. Seven to ten mice were collected for each genotype and timepoint at day three, seven and ten after forced involution. Animals were handled according to approved Vanderbilt Institutional Animal Care and Use Committee protocols.

**Histology, immunofluorescence (IF) and TUNEL analyses**

Paraffin embedded tissues were sectioned at 5uM for both hematoxylin and esosin stained sections and unstained sections used for immunofluorescence (IF) and TUNEL analyses. Npt2b IF was conducted using a custom polyclonal rabbit C-terminal antibody (1:2000, two hours at room temperature) kindly provided by Dr. Fayez Ghishan at the University of Arizona Steele Children's Research Center. Sodium citrate (pH6) was used for antigen retrieval. The secondary antibody was goat anti-rabbit alexa 594 from Invitrogen (A11012). Apoptag TUNEL analyses were conducted as previously described (Bierie et al., 2008). Quantitation of relative TUNEL positive cell counts was determined using three random fields of lobular alveolar structures for each tissue section and three sections from individual mice for each genotype and timepoint. p-values were determined using un-paired t-tests.

### Western and northern blot analyses

Tissues were snap frozen and kept at -80C until protein or RNA were prepared for analysis. Protein was prepared and western blots were run as previously described (Bierie et al., 2008). Primary antibodies including Stat3 (Cell Signaling #9132, 1:1000), p-Stat3 Tyr-705 (Cell Signaling #9131, 1:1000), p53 (Novacastra #NCL-p53-CM5p, 1:500) and β-Actin (Sigma #A-2066, 1:5000) were incubated at 4C overnight. The HRP conjugated secondary antibody was visualized using Amersham ECL (Stat3 and β-actin) or ECL plus (p-Stat3 and p53) reagent from GE Healthcare, UK. RNA was prepared using a standard guanidinium thiocyanate phenol chloroform extraction (Chomczynski and Sacchi, 1987). Northern blots were performed as previously described (Gorska et al., 2003; Gorska et al., 1998). *WAP*, *p53* and *CypA* probes have been previously reported (Gorska et al., 2003; Gorska et al., 1998; Thangaraju et al., 2005). The p53 probe was kindly provided by Dr. Esta Sterneck, Laboratory of Cell and Developmental Signaling, NCI at NIH.

## Results

### WAP-Cre mediated epithelial cell specific deletion of TβRII resulted in accumulation of milk protein and lipid products during mammary involution

We deleted TβRII in mammary epithelial cell populations using WAP-Cre and examined tissues derived from virgin, pregnancy, lactation and involution timepoints. The Rosa26R reporter was used to determine the efficiency of WAP-Cre mediated

recombination at each timepoint used for analysis (data not shown). The results for Rosa26R activation in our WAP-Cre targeted type II TGF-β receptor (TβRII) deficient model [TβRII$^{(WKO)}$Rosa26R] closely paralleled the previously reported expression profile for the WAP-Cre transgene *in vivo* (Wagner et al., 1997). During virgin mammary development we collected tissues at twelve and twenty weeks of age. The virgin timepoints were included due to recent reports that WAP-Cre can mediate recombination in a small subset of virgin mammary progenitor cells during development with a slight increase in the number of cells targeted during estrus (Kordon et al., 1995). However, we did not observe a phenotype in the virgin tissues at either timepoint related to lobular alveolar hyperplasia or aberrant terminal differentiation through analyses conducted using whole mount staining, histological sections and milk gene expression profiles by northern blot. We examined tissues at day fifteen of pregnancy and day three of lactation when WAP-Cre is known to be induced. We did not observe a significant difference in the mammary tissues by whole mount staining and histology or precocious milk gene expression by northern blot analyses at either timepoint. We next examined day one, two, three, seven and ten of involution. We did not observe any differences in morphology at day one or two of involution; however, at day three of involution many of the alveoli in the control tissues had collapsed whereas the alveoli in the TβRII$^{(WKO)}$Rosa26R tissues were predominantly distended (Figure 6A, a-d). Further, we observed a clear difference in milk protein expression by day three of involution (Figure 6B). At this timepoint the TβRII$^{(WKO)}$Rosa26R tissues failed to downregulate WAP protein expression to the extent observed in control tissues.

**Figure 6. Histology and accumulation of WAP protein during early involution.** **A.** Histology from mammary tissues during involution. (a, c, e) Control mammary tissues expressing only the WAP-Cre transgene at day one (a), two (c) and three (e) after forced involution. (b, d, f) TβRII$^{(WKO)}$Rosa26R tissues at day one (b), two (d) and three (f) after forced involution. No differences were noted in histology associated with the first two days of involution. However, by the third day of involution control lobular alveolar structures began to collapse while many lobular alveolar structures in TβRII$^{(WKO)}$Rosa26R tissues at the same timepoint remained distended. **B.** Western blot analysis of WAP protein expression indicated an accumulation in TβRII$^{(WKO)}$Rosa26R tissues when compared with the control tissues at the same timepoint. Ctl, WAP-Cre; KO, TβRII$^{(WKO)}$Rosa26R; L3, lactation day three; D1, involution day one; D2, involution day two; D3, involution day three.

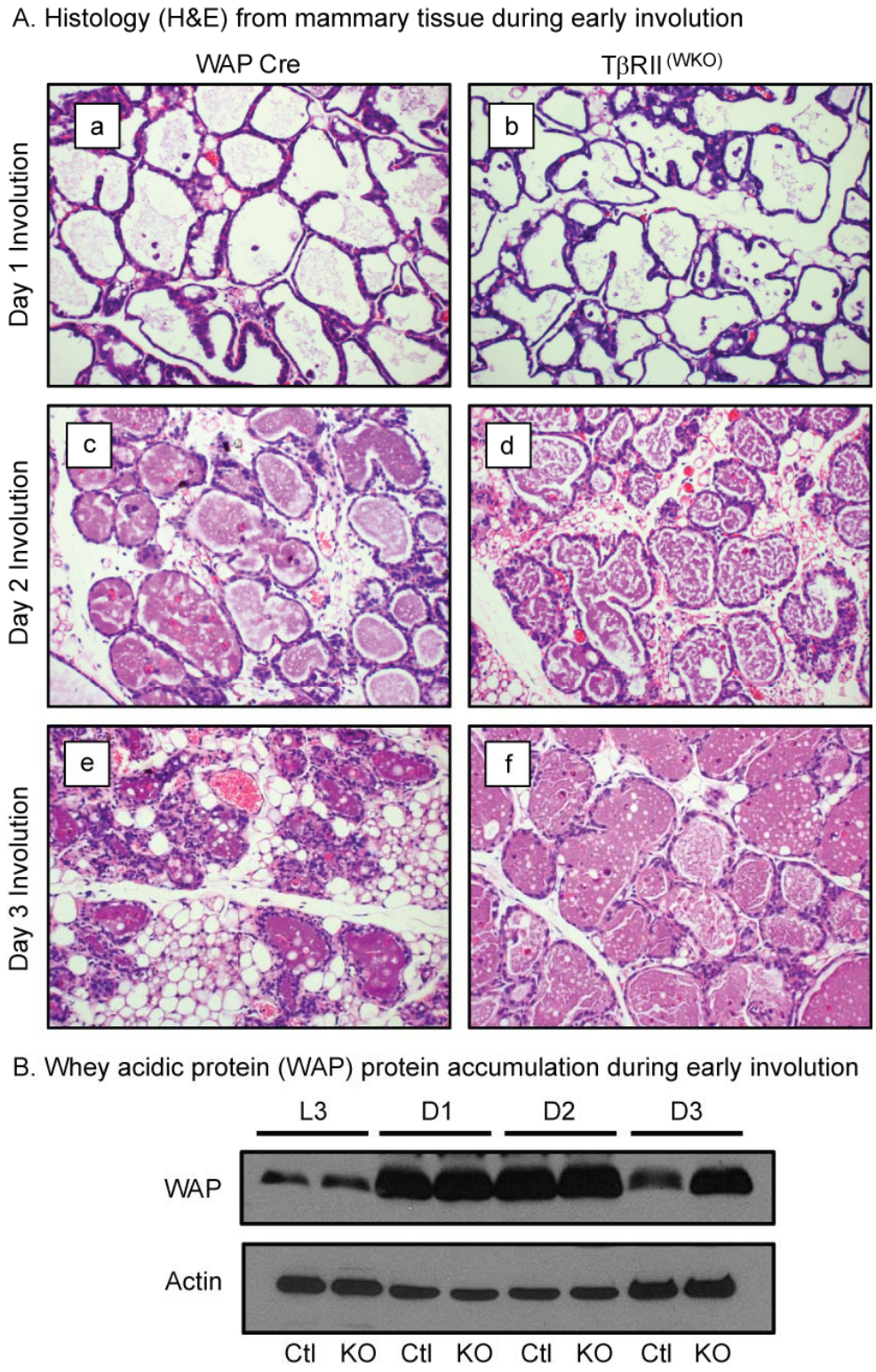


**Figure 6. Histology and accumulation of WAP protein during early involution.**

**Figure 7. Histology during late stages of mammary involution and remodeling. A.** Histology of mammary tissue associated with day seven after forced involution. (a, b) Control mammary tissues expressing only the WAP-Cre. (c, d) TβRII$^{(WKO)}$Rosa26R tissues. At low magnification (a, c) the control tissues appeared largely remodeled with minimal evidence of residual terminally differentiated lobular alveolar structures while TβRII$^{(WKO)}$Rosa26R tissues display an intermediate stage of partial remodeling with many residual expanded lobular alveoli. At higher magnification (b, d), the alveoli in control tissues appear to have returned to a virgin like state while the TβRII$^{(WKO)}$Rosa26R tissues displayed the presence an eosin stained protein component in addition to abundant lipid droplets within the alveolar lumina. **B.** Histology of mammary tissue associated with day ten after forced involution. (a, b) Control mammary tissues expressing only the WAP-Cre. (c, d) TβRII$^{(WKO)}$Rosa26R tissues. The control tissue at this timepoint exhibited nearly complete remodeling at low (a) and high magnification (b). TβRII$^{(WKO)}$Rosa26R tissues at this timepoint remained in an intermediate state of partial involution and remodeling (c). At higher magnification distended alveoli were visible (d), however the expansion was not as prevalent at this timepoint when compared with tissues from day seven of involution.

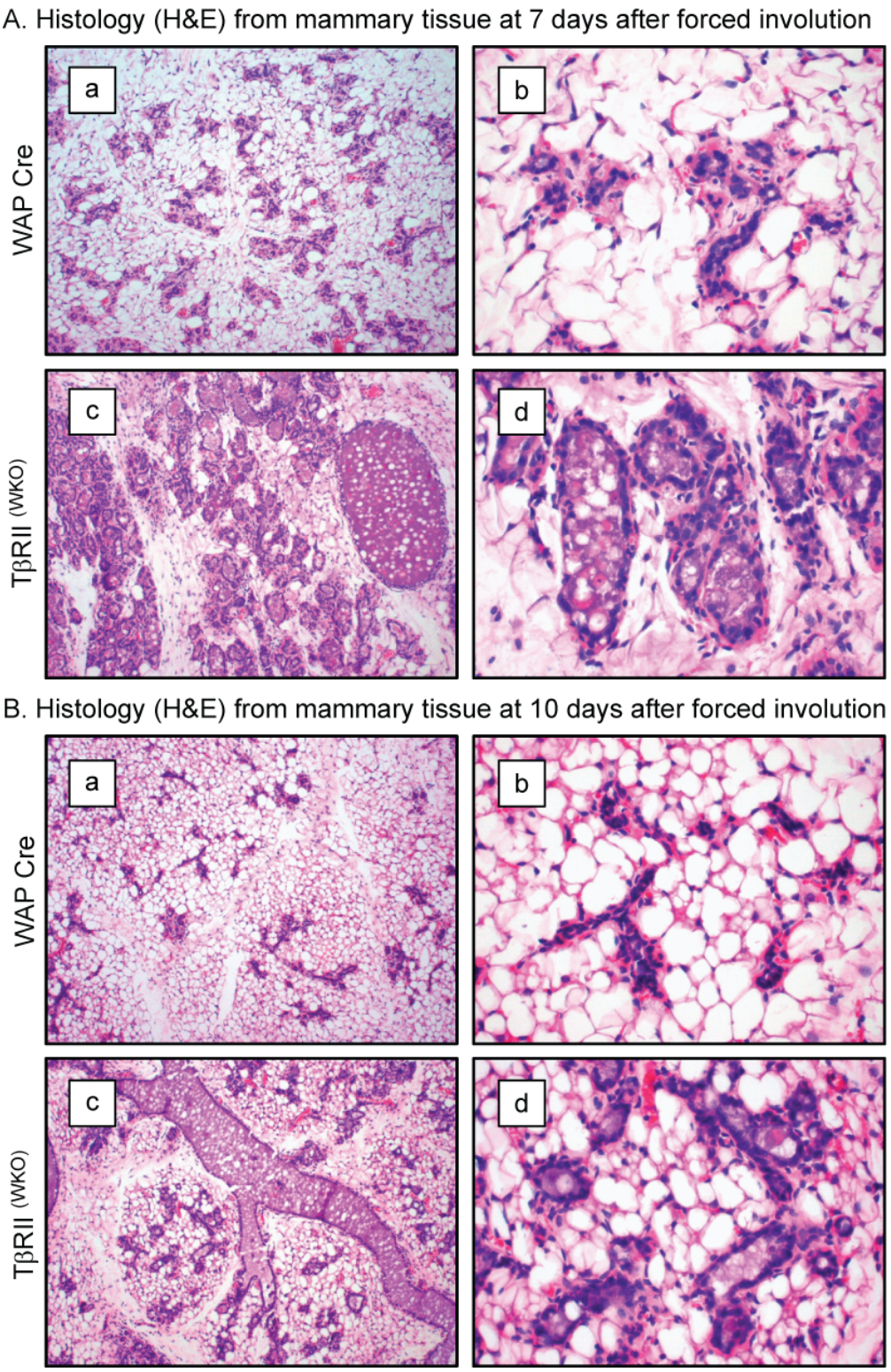


**Figure 7. Histology during late stages of mammary involution and remodeling.**

During later timepoints, at seven and ten days of involution, the alveoli present in TβRII$^{(WKO)}$Rosa26R tissues appeared distended and demonstrated evidence of milk protein and lipid secretion from mammary epithelium in hematoxylin and eosin stained sections. Together, these observations suggested that the TβRII$^{(WKO)}$Rosa26R were resistant to complete involution and the remaining alveoli were actively secreting milk protein and lipid products during late involution (Figure 7).

**Loss of TβRII is associated with differences in the rate of apoptosis during late stages of involution in TβRII$^{(WKO)}$Rosa26R tissues**

The difference in WAP protein expression at day three of involution prompted apoptosis analyses starting at this timepoint, since it was known that the second irreversible stage of mammary involution correlates with the downregulation of milk gene expression. We performed TUNEL analyses on tissues from day three, seven and ten of involution in TβRII$^{(WKO)}$Rosa26R and control mammary tissues (Figure 8). At day three of involution we did not detect a difference in the level of cell death when comparing the two models. However, at day seven and ten of involution we detected a significant increase in the percentage of apoptotic epithelial cells associated with TβRII$^{(WKO)}$Rosa26R tissues when compared to the controls. This observation together with the gross histological analyses suggested that most of the apoptosis was complete in the control tissues by day seven of involution and the process had been delayed upon deletion of TβRII in the mammary epithelium.

**Figure 8. Apoptosis analysis at day three, seven and ten of involution. A.** Apoptag (TUNEL) IHC of mammary tissues during involution. (a, c, e) Control mammary tissues expressing only the WAP-Cre transgene at day three (a), seven (c) and ten (e) after forced involution. (b, d, f) TβRII$^{(WKO)}$Rosa26R tissues at day three (b), seven (d) and ten (f) after forced involution. **B.** Quantitation of Apoptag positive cells at day three (D3), seven (D7) and ten (D10) of involution. Three random apoptotic fields per section were counted with three individual mice per genotype at each timepoint.

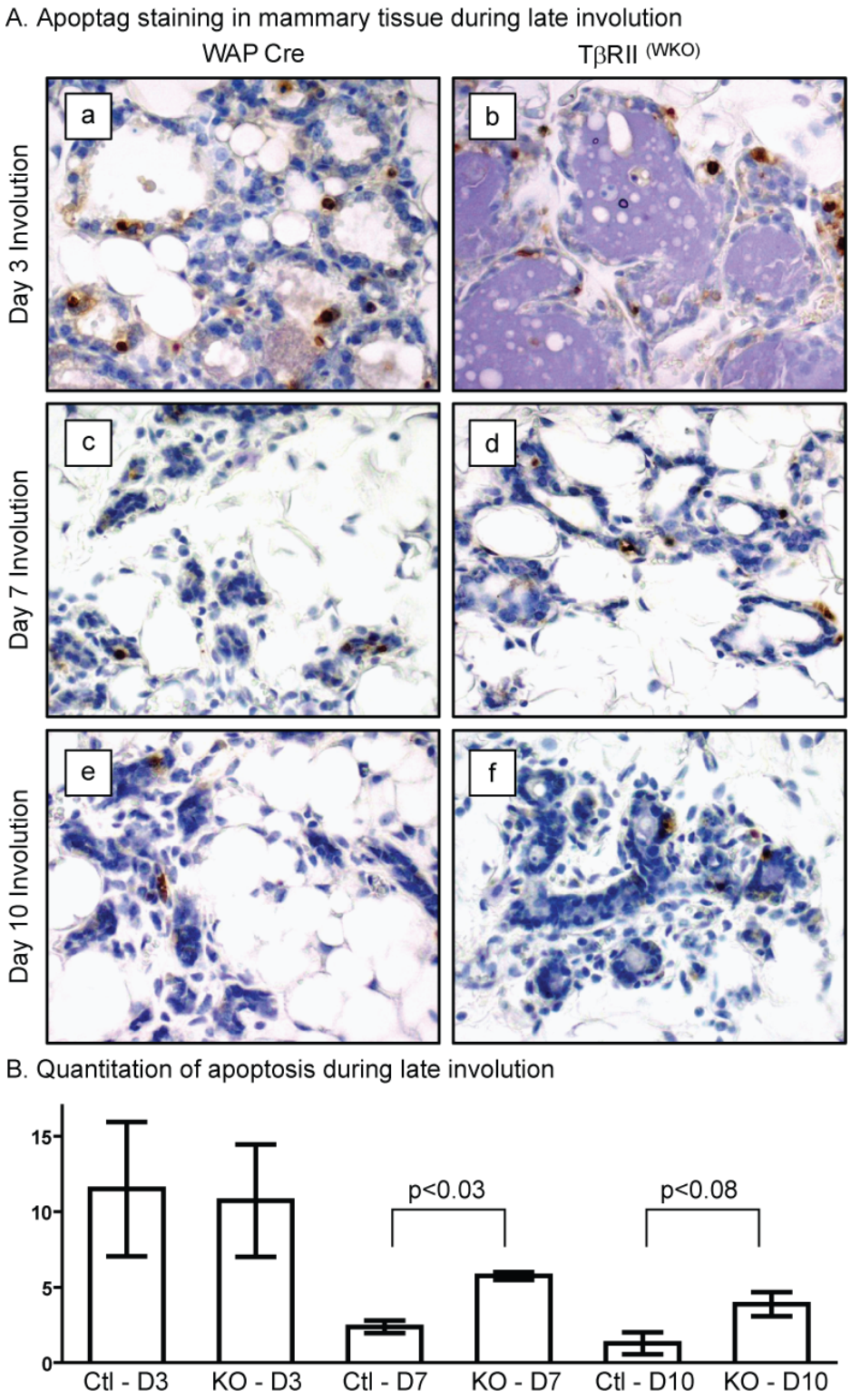


**Figure 8. Apoptosis analysis at day three, seven and ten of involution.**

**Figure 9. Stat3 and p53 expression during late stages of mammary involution. A.** At day three of involution, activated Stat3 (phosphorylation of Tyr-705) was more prevalent in TβRII$^{(WKO)}$Rosa26R tissues. p53 expression was also elevated at this timepoint. Stat3α was more prevalent than Stat3β in both models at this timepoint. β-actin was used as a loading control. Ctl, WAP-Cre; KO, TβRII$^{(WKO)}$Rosa26R. **B.** At seven days of involution Stat3 activation and level of expression is comparable between the control and TβRII$^{(WKO)}$Rosa26R models. Stat3β expression was observed in both models at this timepoint, however the level of p53 protein expression was no longer detectable at a significant level in either model. β-actin was used as a loading control. **C.** At day ten after forced involution, Stat3 activation was significantly reduced in the control and TβRII$^{(WKO)}$Rosa26R models with only one of the three TβRII$^{(WKO)}$Rosa26R models exhibiting an elevated level of Stat3 activation at this timepoint. Stat3α and Stat3β levels were comparable in the control and TβRII$^{(WKO)}$Rosa26R models. p53 protein was not detected at a high level during this timepoint in either model. **D.** p53 mRNA expression analyses by Northern blot revealed an elevated level of expression was maintained in TβRII$^{(WKO)}$Rosa26R tissues at day seven and ten of involution when compared with the WAP-Cre controls. CycA, cyclophillin A was used as a loading control. Ctl, WAP-Cre; KO, TβRII$^{(WKO)}$Rosa26R.

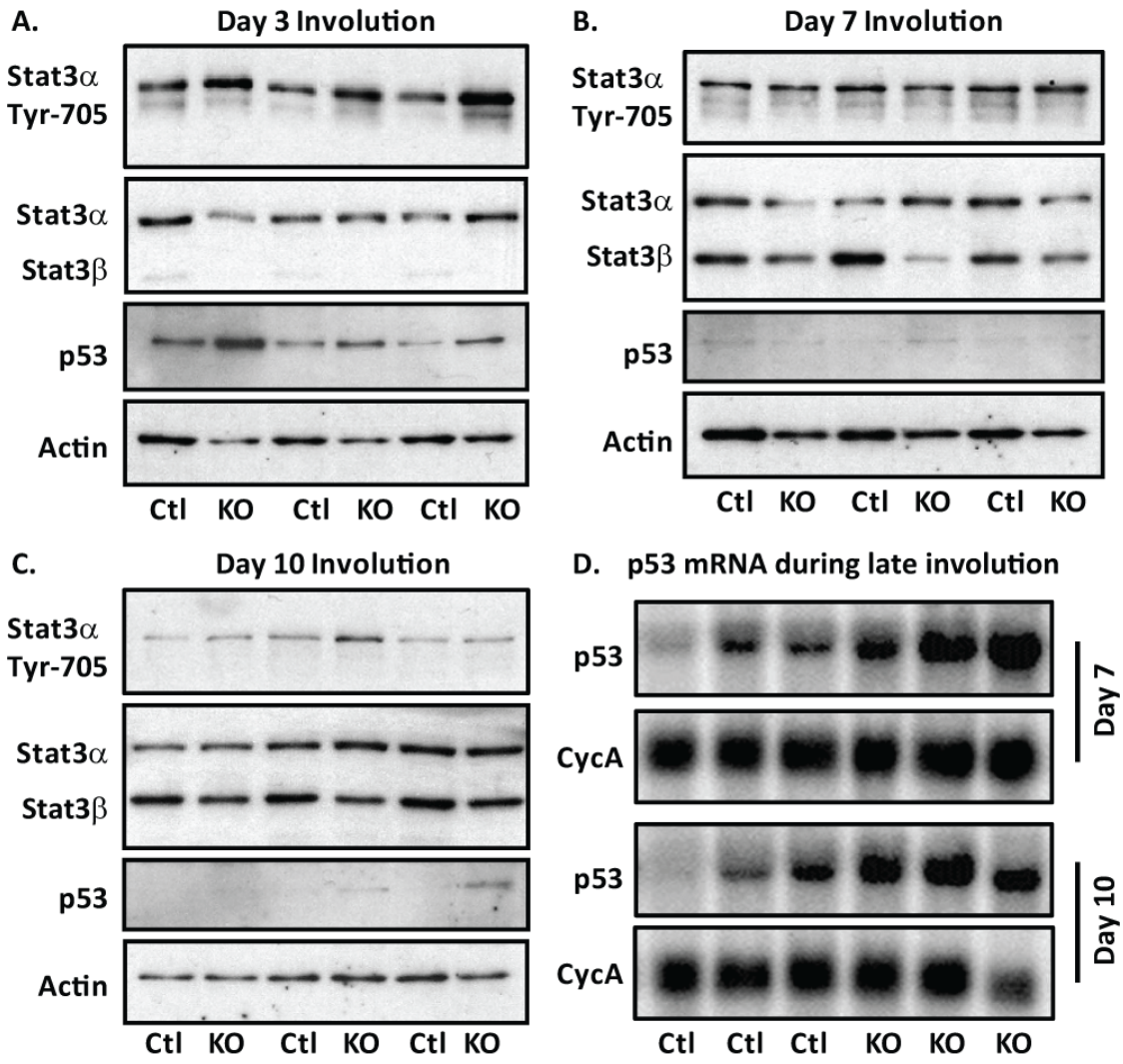


**Figure 9. Stat3 and p53 expression during late stages of mammary involution.**

To determine if major changes in known apoptotic signaling pathways could account for the apparent delay in apoptosis associated with the TβRII$^{(WKO)}$Rosa26R tissues we performed a panel of western and northern blot analyses. Specifically, we examined the expression of Stat3, p-Stat3, p42/44, p-p42/44, MEK1/2, p-MEK1/2, p38, p53, Bax, Bad, Bid, Bim, Bcl-xl, Bcl-2, GP130, CEBPd, Dapk2, Puma, c-jun, jun-D, fos-B, c-fos and cleaved Caspase-3. Interestingly, the only consistent difference that corresponded to the changes observed during late involution timepoints occurred in the pro-apoptotic Stat3 and p53 pathways (Figure 9). Although we were unable to identify a mechanism for the cell survival signals if present, the Stat3 and p53 data indicated that an elevation of pro-apoptotic signaling was present within the TβRII$^{(WKO)}$Rosa26R tissues. Due to the absence of a difference in the rate of apoptosis (Figure 8) and metalloproteinase induction (data not shown) at day three of involution, our results suggested that the elevated pro-apoptotic signals were unable to significantly enhance the progression of involution in TβRII$^{(WKO)}$Rosa26R tissues.

**Na–Pi type IIb co-transporter (Npt2b) protein silencing was not altered during the first phase of mammary involution when TβRII was ablated in mammary epithelium**

The histological examination and differences in the rate of apoptosis, together with the western and northern blot analyses suggested that there may be a delay in committing to the second irreversible phase of involution associated with the TβRII$^{(WKO)}$Rosa26R tissues. However, further analysis was necessary to refine the interpretation. Although we were able to show increased WAP protein accumulation in

TβRII$^{(WKO)}$Rosa26R mammary tissues at day three of involution, the western blot analyses alone did not indicate whether the mammary tissues were actively producing lactation associated gene products. Further, western blot analyses lacked the ability to demonstrate specifically which cells were actively secreting lactation products if present at each timepoint during involution. We felt that it was critical to understand if the TβRII$^{(WKO)}$Rosa26R glands were responding to any of the normal apoptotic stimuli present during involution, or alternatively, if they were resistant to the signals that induce commitment to the second stage of mammary involution. A highly sensitive marker for active lactation, Npt2b, has been used previously as a correlate with lactation status in other systems (Long et al., 2003; Miyoshi et al., 2001; Shillingford et al., 2003). To address this issue, we performed IHF for Npt2b to determine if there was a shift in the downregulation of this lactation marker during the process of involution in our tissues (Figure 10 and 11). We analyzed day three of lactation as a positive control and involution timepoints including day one, two and three after forced weaning. During lactation, no differences were observed in Npt2b expression with regard to intensity or localization (Figure 10A). In the early involution timepoints, at day one through three after forced weaning, there were no observed differences in Npt2b expression (Figure 10B). Notably, at day three of involution (data not shown), Npt2b was not expressed at the apical surface of either the TβRII$^{(WKO)}$Rosa26R or control tissues.

**Figure 10. Analysis of Npt2b expression during lactation and early involution. A.** Expression and localization of Npt2b during lactation. Npt2b was expressed at the apical surface of lobular alveoli whereas no expression was detected in association with ductal epithelium (a, arrow). During lactation, no significant differences were observed when comparing the WAP-Cre (a) and TβRII$^{(WKO)}$Rosa26R tissues (b). **B.** Expression and localization of Npt2b during day one and two of involution. No significant difference in Npt2b localization or level of expression were observed at either timepoint when comparing WAP-Cre and TβRII$^{(WKO)}$Rosa26R tissues. In control and TβRII$^{(WKO)}$Rosa26R tissues no shift in Npt2b silencing was observed that would indicate a significant difference in the initiation of mammary involution. At day three of involution (not shown) Npt2b expression was completely silenced in both models.

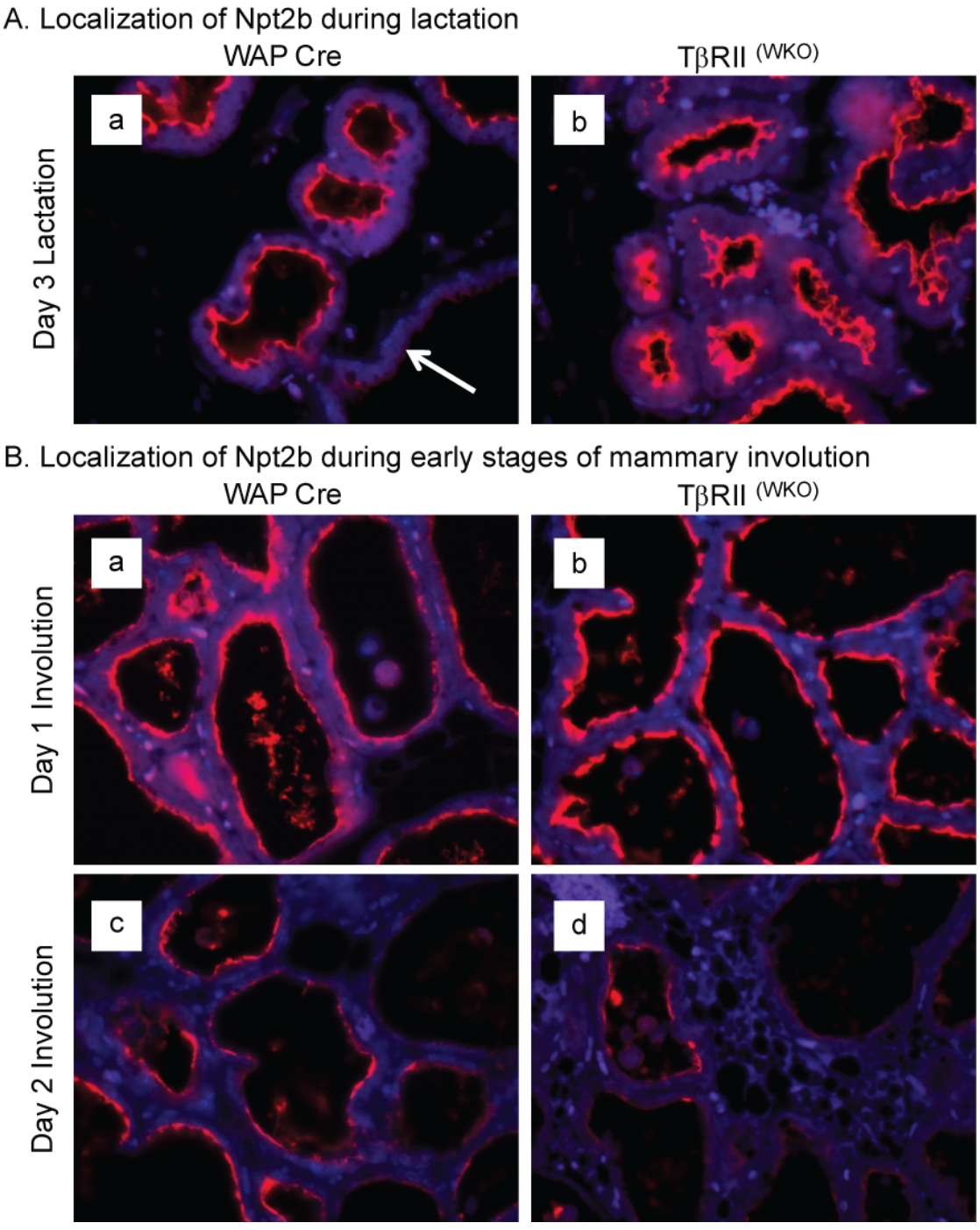


**Figure 10. Analysis of Npt2b expression during lactation and early involution.**

**Figure 11. Re-initiation of a lactogenic phenotype during late stages of mammary involution. A.** Npt2b expression at day ten after forced involution. (a) WAP-Cre control tissues did not display Npt2b expression, while TβRII$^{(WKO)}$Rosa26R tissues (b) continued to express Npt2b at the apical surface of luminal lobular alveolar epithelial cells. **B.** *Whey acidic protein* (*WAP*) mRNA expression during late involution. *WAP* expression was completely silenced in WAP-Cre control tissues by day seven of involution, however it was expressed at a relatively high level in TβRII$^{(WKO)}$Rosa26R tissues. *CypA*, *cyclophillin A* was used as a loading control. **C.** Localization of Npt2b in virgin TβRII$^{(MKO)}$ and MMTV-DNIIR tissues (19 and 20 weeks of age respectively). Hyperplastic terminally differentiated lobular alveolar structures obtained from mice during virgin mammary development expressed Npt2b at the luminal apical epithelial surface in both alternate models of mammary epithelial cell specific TGF-β signaling deficiency.

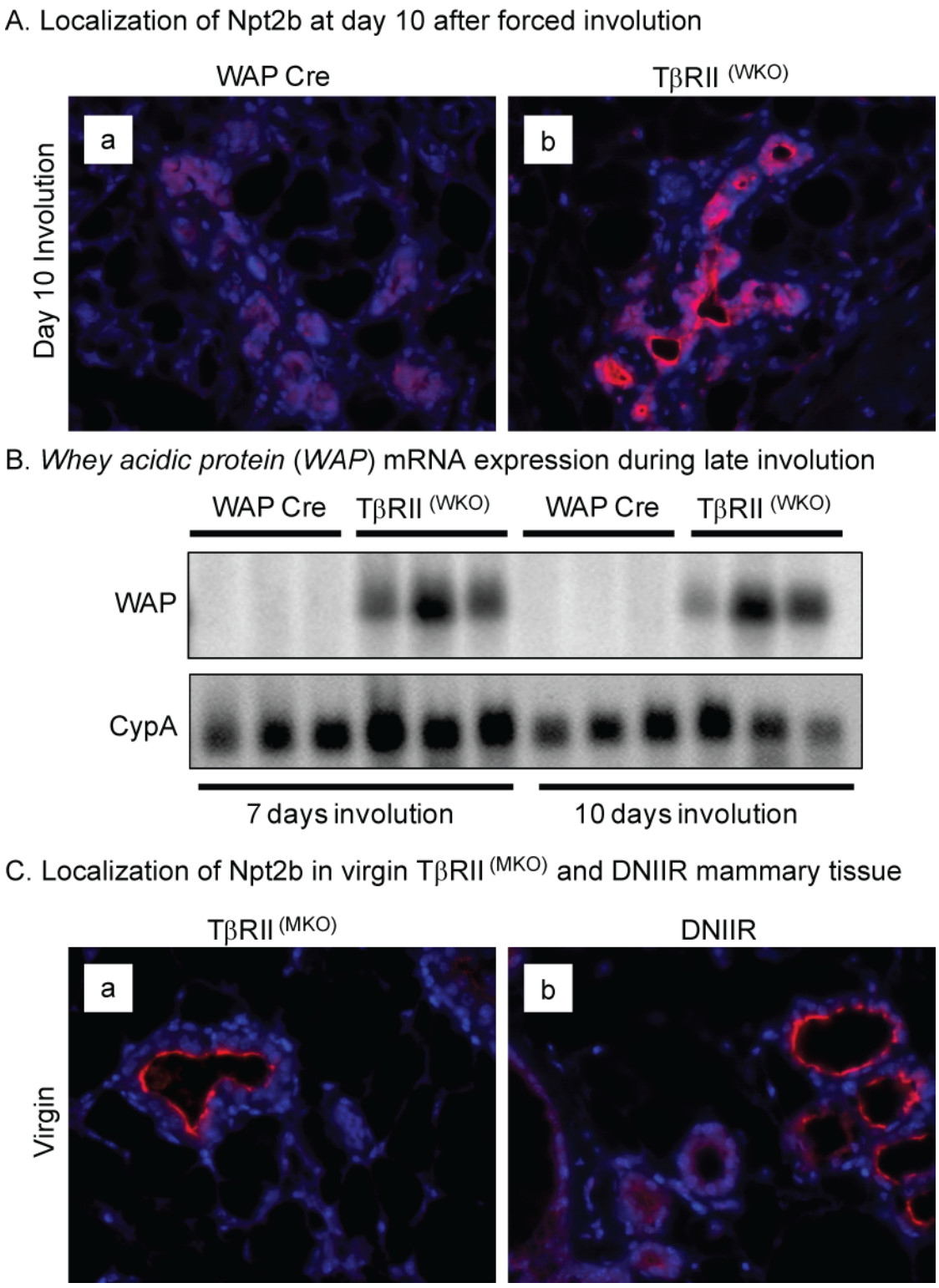


**Figure 11. Re-initiation of a lactogenic phenotype during late stages of mammary involution.**

**Lactation associated Npt2b protein expression was re-initiated in TβRII ablated mammary epithelium during late stages of involution**

Our apoptosis and Npt2b results indicated that the glands lacking TβRII in mammary epithelium were able to respond to apoptotic stimuli through the first three days of involution. The rate of apoptosis and silencing of Npt2b expression did not appear to be significantly altered throughout these timepoints *in vivo*. However, the histology associated with late involution timepoints suggested that the glands were actively producing milk protein and lipid products (Figure 7A and B). Importantly, at day seven and ten of involution there was a clear difference between the models with regard to Npt2b expression (Figure 11A). In the TβRII$^{(WKO)}$Rosa26R tissues, the remaining distended lobular alveolar structures also demonstrated robust Npt2b expression at the apical luminal surface. The mammary alveolar epithelial cells that remained within the gland closely resembled hyperplastic lobular alveolar side-branches previously described as functionally regulated by TGF-β *in vivo* (Chytil et al., 2002; Forrester et al., 2005; Gorska et al., 2003; Gorska et al., 1998).

To further validate the Npt2b results, and to examine the status of milk gene expression, we performed Northern blot analyses for *WAP* mRNA using control and TβRII$^{(WKO)}$Rosa26R tissue during late involution timepoints (Figure 11B). The results indicated that *WAP* gene expression was completely silenced in the control tissues by day seven of involution, whereas the TβRII$^{(WKO)}$Rosa26R tissues maintained expression at a relatively high level through day ten of involution. These results were unexpected due to the complete silencing of Npt2b expression in both control and TβRII$^{(WKO)}$Rosa26R tissues at day three of involution (data not shown). Together, the Npt2b data and *WAP*

gene expression profile indicated that lactation had been re-initiated in the TβRII$^{(WKO)}$Rosa26R tissues during the late stages of mammary involution and remodeling.

**Terminally differentiated mammary epithelium during late stage TβRII$^{(WKO)}$Rosa26R involution resembled hyperplastic terminal differentiation in alternate models of TGF-β signaling deficient mammary tissue**

The localization of Npt2b expressing lobular alveolar structures in the TβRII$^{(WKO)}$Rosa26R tissues appeared morphologically similar to those observed in the previously described TβRII$^{(MKO)}$ and MMTV-DNIIR models. However, Npt2b expression was not previously assessed in these models and this made the direct comparison difficult. We probed the TβRII$^{(MKO)}$ and MMTV-DNIIR models for Npt2b protein expression and found a striking similarity between our TβRII$^{(WKO)}$Rosa26R late involution tissues and the hyperplastic lobular alveolar structures from mammary tissues in the two previously described mouse models (Figure 11C). Together, the results suggested that the late involution phenotype observed in our TβRII model may actually represent both resistance to apoptosis and subsequent hyperplastic expansion with spontaneous differentiation of mammary epithelium as previously described in the TβRII$^{(MKO)}$ and MMTV-DNIIR models. This compound influence is likely, since we have permanently deleted TβRII from the mammary lobular alveolar epithelium starting at mid-pregnancy. As a result, the TβRII deleted epithelial cells may no longer be able to respond to TGF-β dependent suppression of hyperplastic growth or spontaneous terminal differentiation during late involution and remodeling within the mammary

microenvironment. The only substantial difference between our TβRII$^{(WKO)}$Rosa26R model and the MMTV driven lines during late involution and remodeling is the epithelial cell subpopulation targeted by the promoter driving transgene expression in each model. In our WAP-Cre targeted TβRII ablation model, the phenotype should only arise from terminally differentiated lobular alveolar mammary epithelial cells or the previously described parity induced mammary epithelial cell (PiMEC) population (Henry et al., 2004; Wagner et al., 1997). In the MMTV-Cre mediated deletion of TβRII or MMTV-DNIIR models the phenotype may have arisen from the basal, ductal or lobular alveolar cell populations (Andrechek et al., 2000; Wagner et al., 2001). We are currently working to determine whether the mammary epithelial cell populations and mechanisms responsible for the observed terminal differentiation phenotypes are similar or unique to each of the three models of diminished and ablated TGF-β signaling *in vivo*.

## Discussion

Our observations upon ablation of the TGF-β response within mammary epithelium suggested that TGF-β can regulate the duration of post-lactation milk accumulation during the process of involution. We initially considered the effect of TGF-β on these two inter-related processes. In the case of TGF-β dependent regulation of milk gene expression, it has been previously shown that TGF-β dependent Smad signaling can inhibit β-casein production though competition with Stat5 for CBP binding at the *β-casein* gene promoter (Cocolakis et al., 2008). The interaction between Smad3 and Smad4 with CBP prevented Stat5 from co-activating transcription that is dependent

upon functional Stat5/CBP complexes. It was also shown that hydrocortisone, insulin and prolactin treatment significantly blocked TGF-β dependent activation of the 3TP-luc reporter (Cocolakis et al., 2008). Together, these results suggest a mutually antagonistic relationship between the Jak/Stat and TGF-β/Smad signaling pathways with regard to the regulation of transcription. Although this mechanism was clearly demonstrated using a promoter construct *in vitro*, the mechanism may be slightly different in the context of a three dimensional mammary microenvironment. In mammary explant cultures TGF-β signaling was also shown to suppress casein synthesis; however, the data indicated that TGF-β regulated casein secretion without having an effect on transcription (Robinson et al., 1993). In subsequent independent experiments, it was shown that TGF-β stimulation had an impact on the differentiation of mammary epithelial cells through preventing the acquisition of a lactogenic phenotype rather than directly inhibiting production or secretion of existing β-casein protein in mammary organ culture explants (Sudlow et al., 1994). In our studies, the data suggested an intermediate mechanism that does not entirely align with any of the previously described models. During the first two days of involution in our study, Npt2b expression which is dependent upon Jak/Stat signaling, demonstrated no difference in either mouse model and was completely silenced by day three of involution. However, in the TβRII$^{(WKO)}$Rosa26R tissues the expression of Npt2b was re-initiated by day seven of forced involution. Whereas the literature has suggested that lactation may be maintained upon loss of TGF-β signaling, our results indicate that loss of TGF-β signaling can promote re-initiation of lactation rather than prolonged maintenance of this process *in vivo*.

In addition to increased sensitivity to stimuli that may result in re-initiation of a lactation phenotype, our results suggested that there were intrinsic differences that enhanced epithelial cell survival in the presence of elevated pro-apoptotic signaling. In mammary epithelial cells, previously published data has suggested that pro-apoptotic effectors may be activated when cells are stimulated with TGF-β1 (Kolek et al., 2003; Mieth et al., 1990). Using a combination of confocal microscopy and immunoelectron spectroscopy to determine the spatial distribution of pro-apoptotic proteins after TGF-β1 stimulation it was shown that Bax/Bid, caspase-8/Bax/Bid and Bax/VDAC-1 co-localized on the membranes of mitochondria (Kolek et al., 2003). The co-localization of these complexes on the mitochondrial membrane was suggested to represent activation. We had hypothesized that if present, some of the resistance to apoptosis may have been due to alteration of these well characterized apoptotic pathways. However, we were unable to detect changes in caspase-3 cleavage that would indicate an altered balance in the net pro- *versus* anti-apoptotic pathways *in vivo*. Further, we did not detect any changes in the abundance of these pro-apoptotic factors that would be another indication of disrupting the delicate balance of survival and death signals within the mammary epithelium.

In addition, no changes were observed in the expression of GP130 or CEBPd that are thought to be the main activator and effector proteins for the Stat3 signaling pathway during mammary involution respectively. Stat3 has been previously shown to be a dominant player in the initiation of the second irreversible stage of mammary involution (Chapman et al., 1999; Humphreys et al., 2002). Interestingly, our data suggested that Stat3 activation was elevated in TβRII$^{(WKO)}$Rosa26R tissues despite the lack of difference in apoptosis rates or CEBPd elevation at day three of involution. This data correlates

with recent reports demonstrating that TGF-β was able to limit Stat3 activation by IL-6, and IL-6 signaling is known to be an important regulatory ligand for Stat3 activation and progression of involution *in vivo* (Walia et al., 2003; Zhao et al., 2004; Zhao et al., 2002; Zhao et al., 2008). In addition, p53 presence represents a second pro-apoptotic signal that was elevated in the TβRII$^{(WKO)}$Rosa26R tissue at this critical timepoint during mammary involution. This link is also interesting, in light of recent data demonstrating that the p53 and TGF-β pathways may interact to regulate a number of processes *in vivo* (Cordenonsi et al., 2003; Cordenonsi et al., 2007; Dupont et al., 2004). In summary, our data suggests that some of the early mammary involution processes are not entirely dependent upon TGF-β signaling. However, our results also suggest that an unknown TGF-β dependent mechanism is required for efficient commitment to apoptotic cell death and suppression of terminal differentiation that may occur during the second irreversible phase of mammary involution *in vivo*.

CHAPTER III

# TGF-β DEPENDENT REGULATION OF MAMMARY CARCINOMA PROGRESSION AND METASTASIS

## Introduction

The transforming growth factor beta (TGF-β) ligands TGF-β1, TGF-β2 and TGF-β3 are potent regulators of cell behavior, that in addition to the regulation of mammary development, can significantly regulate processes involved in tumor initiation, progression and metastasis (Akhurst and Derynck, 2001; Bierie and Moses, 2006b; Derynck et al., 2001; Derynck and Zhang, 2003). TGF-β signaling pathways are altered in a large number of human cancers including those in the breast (Levy and Hill, 2006). Currently, a diverse repertoire of tumor cell autonomous and tumor cell independent mechanisms for the regulation of carcinoma initiation and progression by TGF-β *in vitro* and *in vivo* have been described (Bierie and Moses, 2006a; Bierie and Moses, 2006b). TGF-β in normal epithelium is known to induce arrest of the cell cycle in G1, and it has been suggested that this cytostatic mechanism is important for the suppression of tumor initiation and early tumor progression (Siegel and Massague, 2003). In later stages of tumor progression, TGF-β signaling in the tumor microenvironment is thought to enhance tumor progression (Akhurst and Derynck, 2001; Bierie and Moses, 2006b). TGF-β stimulation in some normal and carcinoma-associated epithelial cell populations is known to induce an epithelial to mesenchymal transition (EMT) that in the context of a tumor microenvironment may enhance carcinoma cell migration and invasion to promote

metastasis (Akhurst and Derynck, 2001; Bierie and Moses, 2006b). Together these observations suggest that TGF-β functions as a tumor suppressor or tumor promoter depending on the context of stimulation. However, many of the early studies were not able to control for local and systemic influences of exogenous TGF-β expression in the mammary tumor microenvironment.

TGF-β signaling has an effect on many cell types within the tumor microenvironment, and it is clear that some of the regulation occurs through direct control of tumor cells *in vivo*. Attenuation of TGF-β signaling in the mammary epithelium has been shown to result in lobular alveolar hyperplasia and decreased tumor latency in the presence of oncogenic stimuli (Gorska et al., 2003; Lenferink et al., 2003). Attenuation of TGF-β signaling in mammary carcinoma cells also resulted in decreased pulmonary metastasis while activation of the pathway specifically within mammary carcinoma cells increased metastasis (Muraoka-Cook et al., 2006; Siegel et al., 2003). The results obtained using transgenic dominant negative Type II TGF-β receptor attenuation of TGF-β signaling suggested that a significant carcinoma cell autonomous role for TGF-β signaling in breast cancer was the cytostatic suppression of early tumor progression and later promotion of tumor progression through enhanced carcinoma cell invasion and metastasis (Oft et al., 1998; Oft et al., 1996). This dogmatic view of TGF-β signaling was subsequently modified when TβRII expression was completely ablated in mice. It was shown, that attenuation of TGF-β signaling produced results that were different from those obtained with the complete tissue specific ablation of TβRII expression *in vivo* (Forrester et al., 2005). Importantly, when TβRII was completely

ablated in the MMTV-PyVmT mouse model, there was a decrease in tumor latency with a dramatic increase in lung metastases (Forrester et al., 2005).

However, off-target effects of TβRII deletion using the MMTV-Cre transgene included a wasting syndrome and spontaneous morbidity due to currently unknown systemic influences of TGF-β signaling (Forrester et al., 2005). Therefore, a more specific approach was necessary to accurately determine the effect and mechanisms for enhanced tumor growth and metastasis when carcinoma cell TGF-β signaling responses were lost *in vivo*. Therefore to address this issue, using Cre/LoxP technology, with the whey acidic protein promoter driving transgenic expression of Cre recombinase (WAP-Cre) we have now ablated the type II TGF-β receptor (TβRII) expression specifically within mouse mammary alveolar progenitors. Transgenic expression of the polyoma virus middle T antigen under control of the mouse mammary tumor virus enhancer/promoter was used to produce mammary tumors in the absence or presence of Cre ($T\beta RII^{(fl/fl);PY}$ and $T\beta RII^{(fl/fl);PY;WC}$ respectively). The loss of TGF-β signaling significantly decreased tumor latency and increased the rate of pulmonary metastasis. The loss of TGF-β signaling was significantly correlated with increased tumor size and enhanced carcinoma cell survival. In addition, we observed significant differences in stromal fibrovascular abundance and composition accompanied by increased recruitment of F4/80+ cell populations in $T\beta RII^{(fl/fl);PY;WC}$ mice when compared to $T\beta RII^{(fl/fl);PY}$ controls. The recruitment of F4/80+ cells correlated with increased expression of known inflammatory genes including *Cxcl1*, *Cxcl5* and *Ptgs2* (cyclooxygenase-2 [COX-2]). Notably, we also identified an enriched K5(+) dNp63(+) cell population in primary $T\beta RII^{(fl/fl);PY;WC}$ tumors and corresponding pulmonary metastases, suggesting that loss of

TGF-β signaling in this subset of carcinoma cells can contribute to metastasis. Together, our current results indicate that loss of TGF-β signaling in mammary alveolar progenitors may impact tumor initiation, progression and metastasis through regulation of both intrinsic cell signaling and adjacent stromal-epithelial interactions *in vivo*.

## Experimental Procedures

### Mouse models

TβRII$^{(fl/fl)}$ mice were crossed with MMTV-PyVmT, WAP-Cre, MMTV-Cre and Rosa26R$^{(fl/fl)}$ transgenic mice to produce the TβRII$^{(fl/fl);PY}$, TβRII$^{(fl/fl);PY;WC}$, TβRII$^{(fl/fl);PY;WC}$Rosa26$^{(fl/fl)}$ and TβRII$^{(fl/fl);PY;MC}$ lines used for analysis (Andrechek et al., 2000; Chytil et al., 2002; Guy et al., 1992a; Soriano, 1999; Wagner et al., 1997). Mice were housed and handled according to approved IACUC protocols. X-gal staining was performed using standard protocols.

### Preparation of lung whole mounts

Lungs were removed and fixed in 10% NBF overnight at four degrees Celsius. The next day lungs were dehydrated, placed in xylene for one hour then changed to fresh xylene overnight. Lungs were re-hydrated then placed under running tap water for 30 minutes. The tissues were dipped in Mayer's hematoxylin for 2 minutes then washed in running tap water for five minutes. Tissues were de-stained in HCl (fresh 1% v/v from a

12N solution) for 20 minutes, rinsed in running tap water overnight, dehydrated and placed in xylene overnight.

**Immunohistochemistry and immunofluorescence**

Immunohistochemistry (IHC) and immunofluorescence (IF) were conducted using standard protocols (available upon request). All IHC and IF protocols were blocked and incubated in the presence of normal horse serum (Vector Laboratories S-2000). Briefly, smooth muscle actin (Dako U7033), vimentin (Dako U7034) and p63 (Lab Vision MS1081P0; 1:200) IHC was conducted as described in the manufacturer's protocol. IHC for von Willebrand Factor (vWF) was performed with a standard pH 8 EDTA epitope retrieval buffer. IHC and IF for phospho-Histone 3 (UBI 06-570; 1:1000), cytokeratin 5 (Covance PRB-160P; 1:5000 IHC, 1:1000 IF), dNp63 (Santa-Cruz SC-8609; 1:200), cytokeratin 8 (Developmental Studies Hybridoma Bank at the University of Iowa TROMA-1; 1:1000) and smooth muscle actin (IF - Calbiochem CP47, mAb 1A4; 1:1000) was performed using a standard pH 6 sodium citrate buffer. IHC for CD31 (OCT frozen sections) and F4/80 (paraffin embedded) was performed by the Vanderbilt Immunohistochemistry Core Facility. Note: due to the low abundance of dNp63 it was essential to use the blue or red wavelength for IF detection to eliminate background autofluorescence. Values obtained were normalized to total tumor tissue present in each image (histogram values for inverted threshold images obtained from the red channel) and reported as a ratio of the value for specific IHC divided by total tissue present in each image (relative pixel density).

**Apoptag analysis**

Re-hydrated paraffin embedded tissue sections were washed three times for five minutes in PBS, incubated for 20 minutes with 0.3% H2O2, washed three times for five minutes in PBS and then subjected to the rest of the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon S7100) protocol starting with incubation in TdT buffer as described by the manufacturer. Quantitation was performed as described for IHC.

**Protein preparation and blotting**

Protein collection and blotting techniques have been previously described (Forrester et al., 2005), however the following buffer was used for lysis: 50mM Tris pH7.5, 150mM NaCl, 10mM EDTA pH 8.0, 0.2% sodium azide, 50mM NaF and 0.5% NP40; Sigma inhibitor cocktails (P2850, P5726 and 8340) were added at 1:100 fresh. Proteins were prepared for loading by mixing 40ug protein with Laemmli Sample Buffer (Bio-Rad 161-0737) and BME (5% final concentration). Primary antibodies TβRII (Santa Cruz L-21; 1:1000), p-Smad3 (kind gift from Dr. Ed Leof; 1:8000), b-actin (Sigma A-2066; 1:4000); cyclin D1 (Santa Cruz SC-717; 1:1000), cyclin D2 (Santa Cruz SC-181; 1:1000) and Cleaved PARP-1 (Cell Signaling 9544; 1:1000) were incubated on the membranes for 2 hours room temperature. The Smad3 antibody (Zymed 51-1500; 1:500) was incubated on the membranes overnight at four degrees.

**Cytokine antibody array**

Cells were isolated as previously described (Forrester et al., 2005) and cultured in complete medium (5% ABS). Sample collection, incubation and detection were performed as outlined by the manufacturer's protocol (Raybiotech, Inc. AAM-CYT-1000).

**RNA preparation and Real-time PCR**

RNA was collected using Trizol reagent then Dnase treated using the manufacturer's protocols (Invitrogen 15596-018 and Promega M610A respectively). RNA samples were further purified using a RNeasy Mini Kit (Qiagen, 74104). cDNA was prepared using Superscript II reverse transcriptase as described by the manufacturer (Invitrogen, 11904-018). Primers used for SYBR green (Bio-Rad, 170-8882) based Real-time PCR analyses were: *Cxcl1* (Ruddy et al., 2004), *Cxcl5* (Ruddy et al., 2004), *Cxcl16* (Ruddy et al., 2004), *Ccl5* (Lean et al., 2002), *Ccl9* (Lean et al., 2002), *Ccl20* (Lean et al., 2002), *Ptgs2* (Brown et al., 2007), and *18S* F 5'- CAA GAA CGA AAG TCG GAG GTT C -3', *18S* R 5'- GGA CAT CTA AGG GCA TCA CAG -3'. Samples were run on a Bio-Rad iCyclerIQ and the Ct values were subjected to statistical analyses after normalization to 18S and transformation to the median.

## Results

### Loss of TβRII in mammary tumor progenitor cells significantly decreased tumor latency while increasing tumor burden and pulmonary metastases

In order to mediate recombination in the mammary gland we used the WAP gene promoter to drive expression of Cre recombinase *in vivo* (Wagner et al., 1997). In contrast to the MMTV-Cre transgene, which mediates a mosaic deletion in all mammary epithelial cell lineages, the WAP-Cre transgene was used in virgin mice to ablate TβRII expression specifically within hormone responsive alveolar progenitors (Ahmed et al., 2002; Henry et al., 2004). We have used the MMTV-PyVmT transgenic mouse line to induce mammary tumors in our mice in the context of intact or ablated TβRII expression to determine the influence of this signaling pathway on tumor progression and metastasis. The WAP-Cre transgene used in this study targeted the tumor progenitor cell population with exquisite specificity (Figure 12A, a-d). At the earliest sign of hyperplasia and in the solid tumor mass we observed a robust recombination of the Rosa26R reporter allele *in vivo*. We observed a highly significant decrease in tumor latency associated with the $T\beta RII^{(fl/fl);PY;WC}$ mice when compared with the $T\beta RII^{(fl/fl);PY}$ controls (Figure 12B). $T\beta RII^{(fl/fl);PY;WC}$ mice also developed tumors significantly earlier than observed in the $T\beta RII^{(fl/fl);PY;MC}$ model (Figure 12B). However, both TβRII null models had increased lung metastases at 28 days after tumor palpation when compared to the controls (Figure 12C).

**Figure 12. Loss of TGF-β signaling specifically within the mammary tumor precursor cell population significantly decreased tumor latency and promoted progression to metastasis. A.** WAP-Cre mediated Rosa26R activation in MMTV-PyVmT tumors. (a) $T\beta RII^{(fl/fl);PY;WC}Rosa26R^{(fl/fl)}$ whole mount x-gal staining of a mammary tumor 28 days after palpation. Blue staining (arrow) indicates recombination in lobular alveolar lesions. The mammary fat pad (fp) did not show evidence of recombination. (b) $T\beta RII^{(fl/fl);PY;WC}$ whole mount x-gal staining of a mammary tumor 28 days after palpation as a negative control. Large cysts (cy) were a common feature in the distal pre-neoplastic $T\beta RII^{(fl/fl);PY;WC}$ tumor tissue. (c and d) 10uM sections through $T\beta RII^{(fl/fl);PY;WC}Rosa26R^{(fl/fl)}$ whole mount x-gal stained mammary tumor tissue 28 days after palpation. (c) In areas of hyperplasia, recombination was observed (asterisks; light and dark blue stain). Adjacent mammary epithelium surrounding an extended lumen (lu) was negative for reporter gene expression (arrow). (d) Solid tumor tissue (T) demonstrated evidence of efficient recombination whereas adjacent stroma (S) was negative. **B.** Kaplan-Meier curves of the time until tumor palpation in WAP-Cre and MMTV-Cre mediated TβRII conditional null MMTV-PyVmT tumors. WAP-Cre dependent loss of TβRII in MMTV-PyVmT tumors significantly decreased tumor latency. The $T\beta RII^{(fl/fl);PY;WC}$ mice developed palpable tumors with a median time of 31 days whereas the $T\beta RII^{(fl/fl);PY}$ control mice had a median time to tumor palpation of 44 days (n=30 for each genotype; $p<0.0001$). MMTV-Cre dependent loss of TβRII in MMTV-PyVmT tumors also significantly decreased tumor latency similar to results from a previous study(14). The $T\beta RII^{(fl/fl);PY;MC}$ mice developed palpable tumors at 36 days whereas the $T\beta RII^{(fl/fl);PY}$ control mice had a median time to tumor palpation of 45 days (n=30 for each genotype; $p<0.0001$). Interestingly, when TβRII was deleted using WAP-Cre, palpable tumors were detected earlier than when using MMTV-Cre to mediate deletion (n=30 for each genotype; $p<0.0001$). The difference in littermate control groups was not significant (n=30 for each group). Significance of data represented in the Kaplan-Meier curves was determined using Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests for statistical significance. **C.** Visible lung metastases in whole mount stains 28 days after tumor palpation. A significant increase in the number of visible metastases occurred in the $T\beta RII^{(fl/fl);PY;WC}$ and $T\beta RII^{(fl/fl);PY;MC}$ models when compared to the $T\beta RII^{(fl/fl);PY}$ controls (n=30 for each genotype; $p<0.005$ and $p<0.0005$ respectively). The $T\beta RII^{(fl/fl);PY}$, $T\beta RII^{(fl/fl);PY;WC}$ and $T\beta RII^{(fl/fl);PY;MC}$ models had 0.9 (+/- 0.3 SEM), 5.1 (+/-1.5 SEM) and 4.4 (+/- 1.0 SEM) metastases respectively. There was not a significant difference when comparing the $T\beta RII^{(fl/fl);PY;WC}$ and $T\beta RII^{(fl/fl);PY;MC}$ models to each other (n=30 for each genotype). Significance for metastasis data was determined using the Wilcoxon signed rank test. **D.** Total body weight at the time of sacrifice demonstrated a significant increase in tumor burden associated with the $T\beta RII^{(fl/fl);PY;WC}$ and $T\beta RII^{(fl/fl);PY;MC}$ models when compared with $T\beta RII^{(fl/fl);PY}$ controls (n=20 for each genotype; $p<0.0001$ and $p<0.0005$ respectively using un-paired t-tests). The mean body weight in the $T\beta RII^{(fl/fl);PY;WC}$ model was 32.75g (+/- 0.99) and the $T\beta RII^{(fl/fl);PY;MC}$ model had a mean body weight of 30.26g (+/- 0.88) while the mean body weight in the $T\beta RII^{(fl/fl);PY}$ control mice was 24.6g (+/- 0.6).

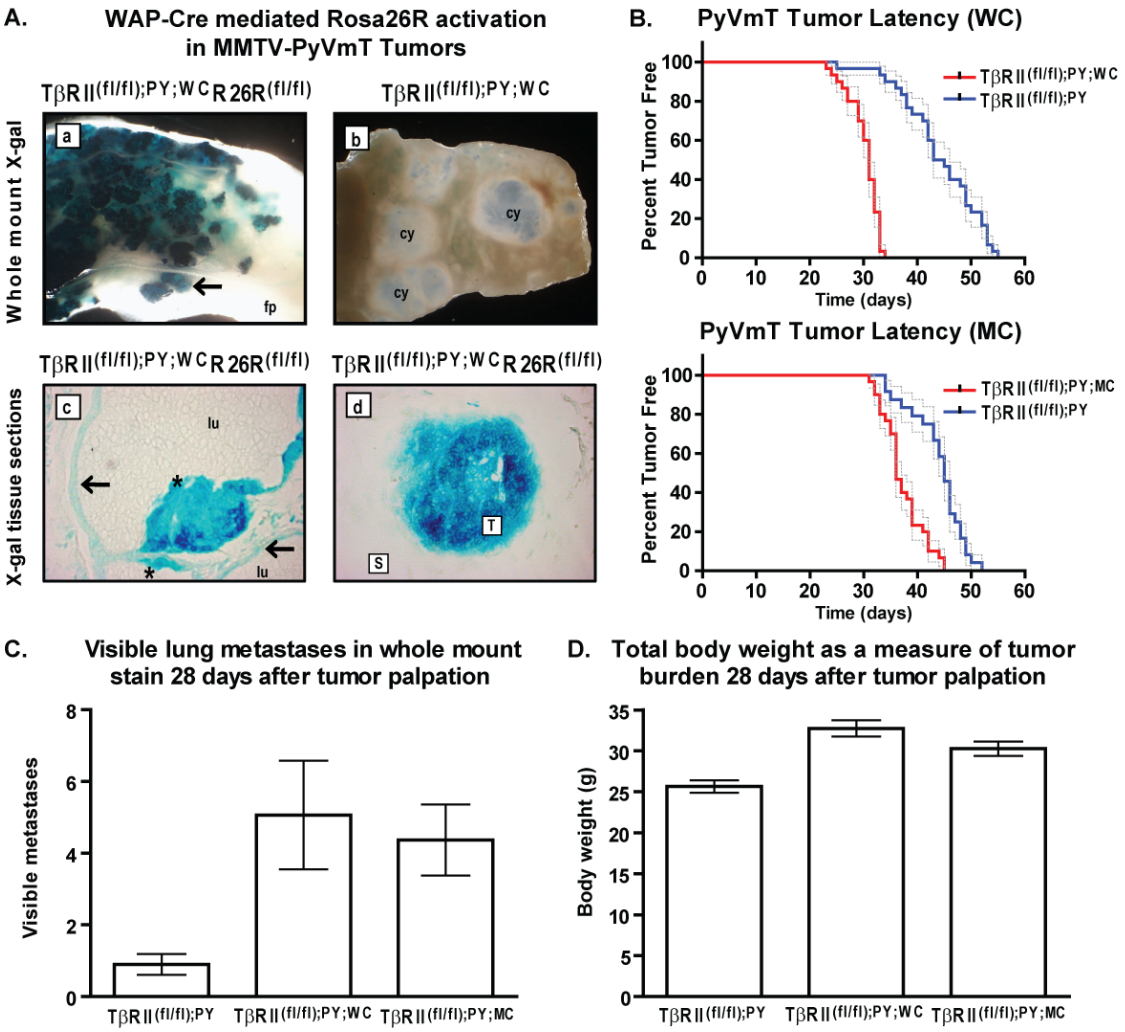


**Figure 12. Loss of TGF-β signaling specifically within the mammary tumor precursor cell population significantly decreased tumor latency and promoted progression to metastasis.**

In this study, it was necessary to sacrifice the mice at 28 days after tumor palpation as opposed to 45 days after tumor palpation in the previous study (Forrester et al., 2005), due to the exceptionally large size of the conditional TβRII null tumors at this time point in the pure FVB background (n>12). At this timepoint, the number of metastases could be quantified by counting the lesions in lung whole mounts rather than using lung weights as previously described (Forrester et al., 2005). The number of metastases in the WAP-Cre and MMTV-Cre models was significantly higher than the controls. However, there was no difference in the number of metastases when comparing the $T\beta RII^{(fl/fl);PY;WC}$ and $T\beta RII^{(fl/fl);PY;MC}$ models to each other (Figure 12C). Further, the total body weight as a measure of tumor burden at the time of sacrifice, was significantly higher in $T\beta RII^{(fl/fl);PY;WC}$ and $T\beta RII^{(fl/fl);PY;MC}$ mice when compared with the $T\beta RII^{(fl/fl);PY}$ controls (Figure 12D).

**Loss of TβRII increased the abundance of cystic mammary pre-neoplastic hyperplasias, solid tumor tissue, pseudo-papillary structures and moderate to well-differentiated extravascular pulmonary metastases**

In the absence of TβRII we found that distal pre-neoplastic hyperplasias present in the MMTV-PyVmT tumors were significantly expanded (Figure 13A, a-c). The hyperplastic growth predominantly involved lobular alveolar epithelium that formed well-differentiated lobular alveoli with distended lumina. The hyperplastic alveoli were often filled with a secretory product that appeared to have an abundant protein composition as indicated by eosin staining.

**Figure 13. TGF-β regulates the expansion and differentiation of pre-neoplastic, solid and pseudo-papillary lesions within primary mammary tumor tissues.** Histopathological analysis of tumor tissues derived from the $T\beta RII^{(fl/fl);PY}$, $T\beta RII^{(fl/fl);PY;WC}$ and $T\beta RII^{(fl/fl);PY;MC}$ models revealed differences in three main mammary tumor compartments. **A.** The distal portion of all MMTV-PyVmT mammary tumor tissues had pre-neoplastic lobular-alveolar mammary hyperplasias (mammary intra-epithelial neoplasia; MIN) with small foci of carcinoma in situ (a-c). One of the most striking histopathologic differences among the models was the presence of large cysts in hyperplastic areas associated with the $T\beta RII^{(fl/fl);PY;WC}$ and $T\beta RII^{(fl/fl);PY;MC}$ mice (cyst, C; hyperplasia, h; lumen, lu). The cysts often contained proteinaceous material. Adjacent and proximal to the MIN region all the tumor models had a mix of solid carcinoma in situ and invasive adenocarcinoma (d-f). Mammary tumor tissues associated with the $T\beta RII^{(fl/fl);PY;WC}$ and $T\beta RII^{(fl/fl);PY;MC}$ models were more differentiated than the $T\beta RII^{(fl/fl);PY}$ controls as determined by the increased frequency of small tubular gland-like structures throughout the solid tumor compartment (yellow arrows). The fibrovascular stroma was more abundant in the $T\beta RII^{(fl/fl);PY;WC}$ and $T\beta RII^{(fl/fl);PY;MC}$ models when compared with the $T\beta RII^{(fl/fl);PY}$ controls (white arrows). Large regions of pseudo-papillary mammary hyperplasia were also observed in all three tumor models (g-i). The pseudo-papillary regions associated with the $T\beta RII^{(fl/fl);PY;WC}$ and $T\beta RII^{(fl/fl);PY;MC}$ models were more cystic than the $T\beta RII^{(fl/fl);PY}$ controls (asterisks). **B.** The lung metastases in all three models had a moderate to well-differentiated morphology with abundant lobular alveolar structures (a-c, arrows). Lung parenchyma (lp).

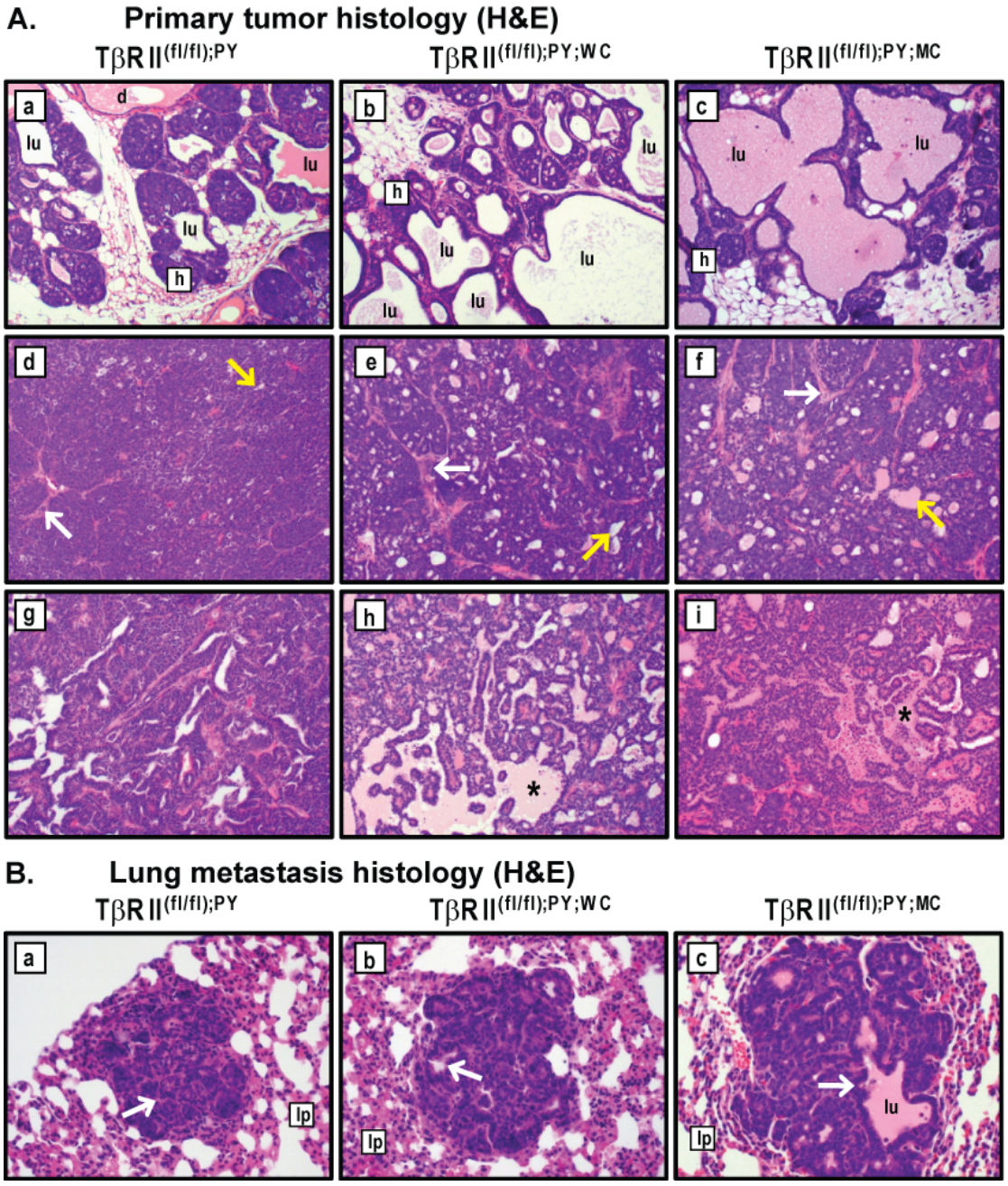


**Figure 13. TGF-β regulates the expansion and differentiation of pre-neoplastic, solid and pseudo-papillary lesions within primary mammary tumor tissues.**

The solid TβRII$^{(fl/fl);PY;WC}$ and TβRII$^{(fl/fl);PY;MC}$ tumor tissues demonstrated a moderate to well-differentiated morphology with tubular gland-like structures that were less abundant in the TβRII$^{(fl/fl);PY}$ controls (Figure 13A, d-f). The solid TβRII$^{(fl/fl);PY;WC}$ and TβRII$^{(fl/fl);PY;MC}$ tumor tissues also demonstrated an expansion of the stromal compartment when compared to the TβRII$^{(fl/fl);PY}$ controls (Figure 13A, d-f). All three tumor models had an abundant pseudo-papillary component, however TβRII$^{(fl/fl);PY;WC}$ and TβRII$^{(fl/fl);PY;MC}$ pseudo-papillary tissues were more cystic than in the TβRII$^{(fl/fl);PY}$ controls (Figure 13A, g-i). The lung metastases in all three models had a moderate to well-differentiated morphology with abundant lobular alveolar structures (13B, a-c).

**In the absence of TβRII, mammary carcinoma cells exhibit enhanced tumor cell survival**

In order to address the mechanisms for enhanced tumor growth and metastasis observed when TβRII was lost in carcinoma cells, we used the highly specific TβRII$^{(fl/fl);PY;WC}$ model in comparison with TβRII$^{(fl/fl);PY}$ controls. The abundant tumor volume visible by gross physical examination and histological analyses indicated that the TβRII$^{(fl/fl);PY;WC}$ tumor tissues grew faster than those in the TβRII$^{(fl/fl);PY}$ controls. This led us to hypothesize that the carcinoma cells had a difference in the rate of proliferation or cell survival. Using phospho-histone 3 as a marker of mitosis, we were able to determine that there was not a significant difference in the rate of proliferation within individual proliferative cell clusters when TβRII was ablated in the mammary carcinoma cells (Figure 14A, a and c).

**Figure 14. TGF-β signaling promotes carcinoma cell death in primary mammary tumor tissues. A.** Primary tumor proliferation and apoptosis were analyzed using phospho-histone 3 (pH3) and Apoptag immunohistochemistry (IHC) respectively. IHC for pH3 revealed clusters of carcinoma cells in mitosis (a and c, brown nuclei). The TβRII$^{(fl/fl);PY}$ and TβRII$^{(fl/fl);PY;WC}$ models both demonstrated proliferating cells in close proximity to the adjacent fibrovascular stroma. Proliferation within the stromal compartment was not observed at a significant level in either tumor model. Apoptag labeling and immunohistochemistry was performed to determine the relative level of apoptotic cell death in the primary tumor tissues (b and c). Quantitation of proliferation and apoptosis in primary tumor tissue IHC was performed (bar graphs). No statistically significant difference was observed when the number of pH3+ cells from random proliferative clusters were quantified in the TβRII$^{(fl/fl);PY}$ tumor tissues and compared to those from the TβRII$^{(fl/fl);PY;WC}$ mice (65.4 +/- 6.2 SEM *versus* 61.2 +/- 7.6 SEM cells per field of view respectively, un-paired t-test; n=6 individual tumors for each genotype and 3 random fields analyzed per tissue section). Apoptag labeling and IHC revealed a significant decrease in apoptosis associated with the TβRII$^{(fl/fl);PY;WC}$ tumor tissues when compared to the TβRII$^{(fl/fl);PY}$ controls (median transformed mean pixel density was 0.65 +/- 0.08 SEM *versus* 1.13 +/- 0.16 SEM respectively; $p<0.01$, un-paired t-test; n=6 individual tumors for each genotype and 3 random fields analyzed per tissue section). **B.** Analysis of TβRII expression and Smad3 activation in TβRII$^{(fl/fl);PY}$ and TβRII$^{(fl/fl);PY;WC}$ mammary tumor tissues. TβRII protein was efficiently deleted in TβRII$^{(fl/fl);PY;WC}$ tumor tissues and this correlated with a decrease in Smad3 phosphorylation (pSmad3). Residual TβRII expression identified by Western blot analysis was likely due to the presence of non-epithelial cell populations within the tumor microenvironment. Total Smad3 levels were not altered in the TβRII$^{(fl/fl);PY;WC}$ model when compared to the TβRII$^{(fl/fl);PY}$ controls. **C.** Cyclin D1 and D2 (annotated Ccnd1 and Ccnd2) expression was significantly higher in the TβRII$^{(fl/fl);PY}$ control tissues. This positively correlated with cyclin A and cyclin B1 expression (data not shown). beta-Actin (Actβ) was used as a loading control. **D.** PARP-1 cleavage was more abundant in control tissues. Typical caspase dependent and atypical caspase independent cleavage products were observed (bands at 89 kDa and 60 kDa, respectively). **E.** Death associated protein kinase 2 (Dapk2) expression was consistently higher in TβRII$^{(fl/fl);PY}$ control tumor tissues. beta-Actin (Actβ) was used as a loading control.

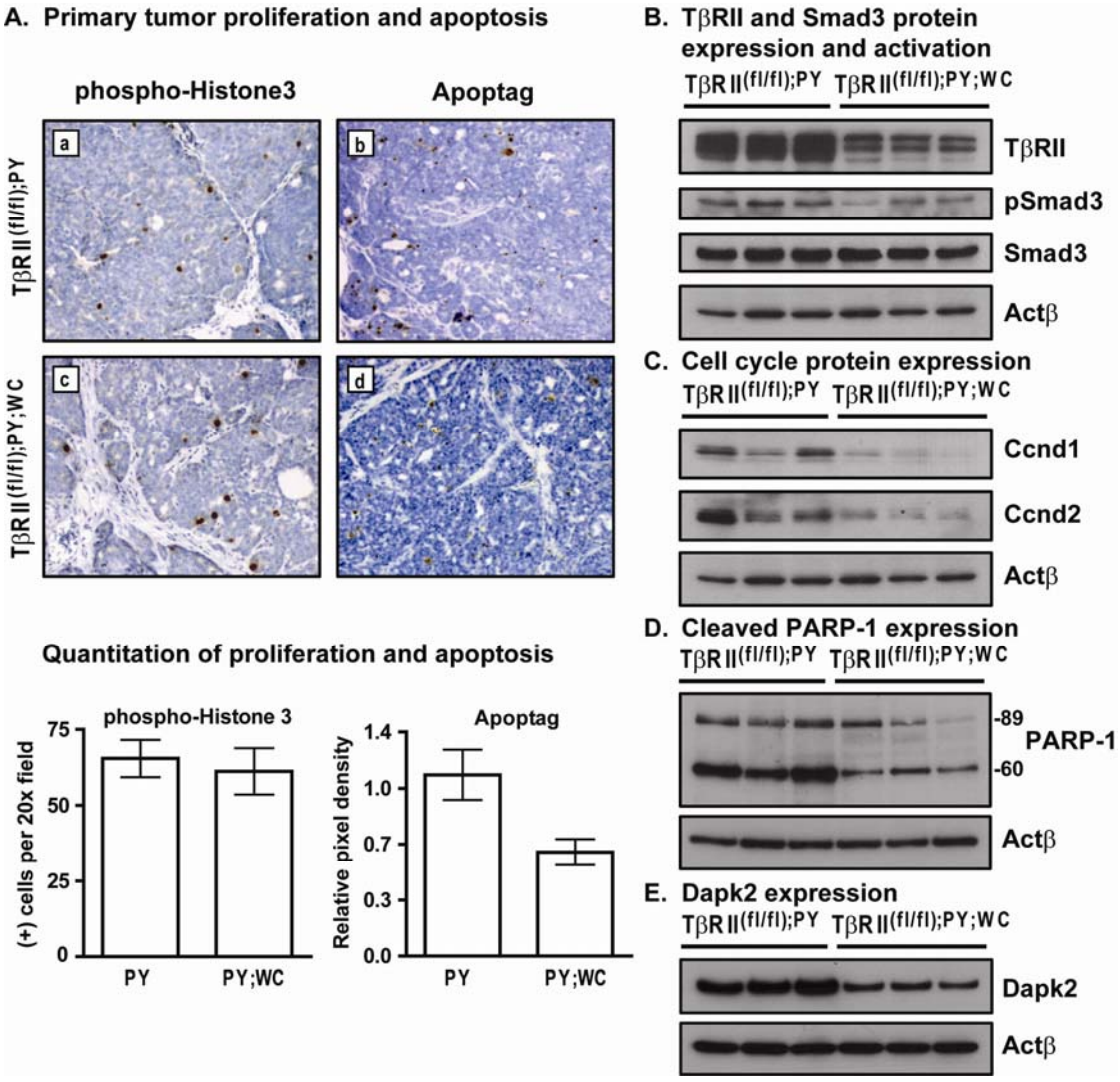


**Figure 14. TGF-β signaling promotes carcinoma cell death in primary mammary tumor tissues.**

**Figure 15. Consolidation of IHC and Western blot proliferation data.** Relative proliferation levels were approximated using the number of mitotic cells (phospho-histone 3 positive nuclei) per proliferative cluster in a microscopic field of view (a) and alternatively by comparing total protein expression of cell cycle associated gene products (Cyclins) in tumor tissue lysates (b). In proliferative clusters (yellow circles), analyses conducted to determine the number of proliferative cells per microscopic field of view (a, white circles surrounding proliferative clusters) resulted in an equal number of proliferative cells per cluster when comparing the two models. In this simplistic illustration (a) the experimental result would be 4 cells per cluster in both tumor models. However, when an equal amount of total tumor tissue was homogenized for total protein analysis by Western blot (b, white circles surrounding proliferative clusters) the result indicated a higher percentage of total tumor tissue was actively cycling in $T\beta RII^{(fl/fl);PY}$ tumor tissues when compared with those in the $T\beta RII^{(fl/fl);PY;WC}$ model. In the case of this simple illustration (b) the result indicates a greater relative percentage of cell cycle dependent protein expression per gram of tissue in the $T\beta RII^{(fl/fl);PY}$ tumors when compared to the $T\beta RII^{(fl/fl);PY;WC}$ model. Together, this concept along with the increased rate of apoptosis observed in the $T\beta RII^{(fl/fl);PY}$ controls by Apoptag staining, increased PARP-1 cleavage in the $T\beta RII^{(fl/fl);PY}$ controls determined by Western blot analyses, and abundant previously published data demonstrating a pro-apoptotic role for TGF-β signaling in mammary epithelium suggest that the $T\beta RII^{(fl/fl);PY;WC}$ carcinoma cells have enhanced cell survival characteristics when compared with those in the $T\beta RII^{(fl/fl);PY}$ control tumor tissues.

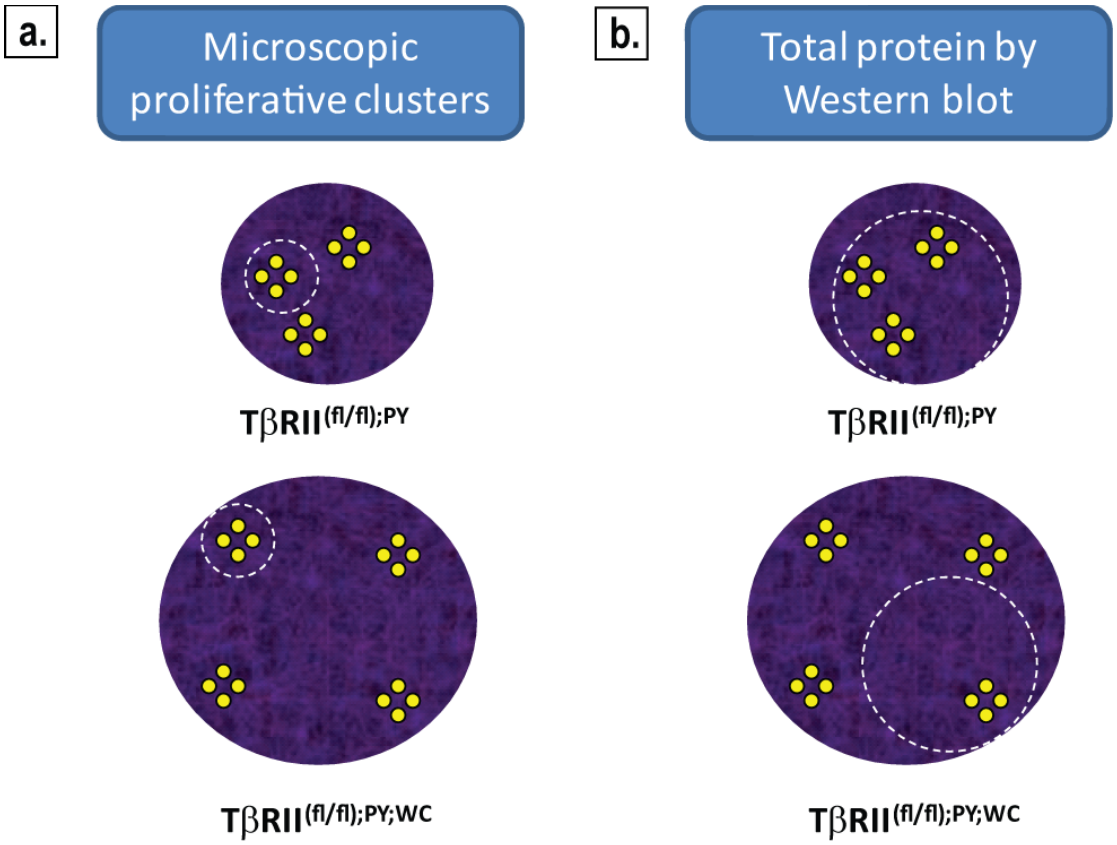


**Figure 15. Consolidation of IHC and Western blot proliferation data.**

Interestingly, the proliferation in both models was predominantly localized within carcinoma cells adjacent to the fibrovascular stroma whereas no significant proliferation was observed in the stromal compartment. Conversely, we found that the TβRII$^{(fl/fl);PY}$ tumor tissues had an increased rate of apoptosis when compared with tissues from the TβRII$^{(fl/fl);PY;WC}$ model (Figure 14A, b and d). When quantified, the relative increase in apoptosis associated with the control tumors was significant (Figure 14A, bar graph).

In order to further assess the status of total proliferation within the tumor tissue rather than within microscopic proliferative clusters we examined the expression of TβRII, Smad3 and p-Smad3 protein (Figure 14B) in addition to several common cell cycle markers including cyclin D1, cyclin D2, cyclin D3, cyclin A, cyclin B1, Cdk2 and Cdk4 (Ccnd1 and Ccnd2, Figure 14C). The control tissues had a significantly higher level of cyclin D1, cyclin D2, cyclin A and cyclin B1 expression without a corresponding change in cyclin D3, Cdk2 or Cdk4 (data not shown for cyclin A, cyclin B1, cyclin D3, Cdk2 and Cdk4). This suggested that a greater percentage of the cells in the TβRII$^{(fl/fl);PY}$ control tissue were actively proliferating (Figure 15). We subsequently examined the expression of cleaved PARP-1 protein as an indicator of caspase activity in our tumor tissues. The control tissues had more cleaved PARP-1 expression than TβRII$^{(fl/fl);PY;WC}$ tissues (Figure 14D). Interestingly, the presence of both typical and atypical PARP-1 cleavage products suggest that both caspase dependent and independent pathways together contribute to the increased cell death associated with TβRII$^{(fl/fl);PY}$ tumor tissues *in vivo* (Bey et al., 2007). Further, the death associated protein kinase 2 (Dapk2) was consistently upregulated in TβRII$^{(fl/fl);PY}$ tissues (Figure 14E), suggesting that resistence to cell death by autophagy is a factor worth consideration in the TβRII$^{(WKO);PY}$ model.

**TGF-β signaling in mammary carcinoma cells can regulate the adjacent fibrovascular stroma during tumor progression**

In the tumor H&E sections, a reactive stroma was observed in the invasive tissues at 28 days after tumor palpation in the TβRII$^{(fl/fl);PY}$ and TβRII$^{(fl/fl);PY;WC}$ tumor models (Figure 16A, a and b). Notably, this reactive stroma was detected in TβRII$^{(fl/fl);PY;WC}$ tissues as early as nine days after tumor palpation (Figure 16A, c). In addition, we observed an expansion of the stromal fibroblast cell compartment in TβRII$^{(fl/fl);PY;WC}$ tumors when compared to TβRII$^{(fl/fl);PY}$ controls (Figures 12A, d-f and 13A, a-d). However, it was not clear if there were phenotypic differences in the stromal fibroblasts associated with the alternate tumor models. Interestingly, most of the stroma in both models expressed vimentin (Figure 16B, a and b), however the stroma in TβRII$^{(fl/fl);PY;WC}$ tumors also had a high level of smooth muscle actin (SMA) expression (Figure 16B, c and d). In the TβRII$^{(fl/fl);PY}$ control tumor tissues, SMA expression was predominantly localized in the stroma around the outer margin of the tumor. In contrast, the TβRII$^{(fl/fl);PY;WC}$ model had abundant SMA expression in stromal cells adjacent to the carcinoma lobules throughout the tumor tissue.

The abundant stroma in TβRII$^{(fl/fl);PY;WC}$ tumors correlated with vascular structures in H&E sections, and we hypothesized that the abundant fibrovascular stroma may have been due to a general increase in angiogenesis. However, the endothelial cell component of the fibrovascular network did not increase in abundance when TβRII was deleted in the carcinoma epithelium. To determine the relative vascular contribution within the tumor microenvironment we performed immunohistochemistry for von Willebrand Factor (vWF) and CD31.

**Figure 16. TGF-β signaling within mammary carcinoma cell regulates the adjacent fibrovascular stroma. A.** Invasive regions within $T\beta RII^{(fl/fl);PY}$ and $T\beta RII^{(fl/fl);PY;WC}$ tumor tissues were often associated with a reactive tumor stroma. Tumor invasion with a reactive stroma was observed in both models 28 days after palpation (a and b). However, in the $T\beta RII^{(fl/fl);PY;WC}$ model invasion associated with a reactive stroma was observed as early as 9 days after tumor palpation (c). Vimentin expression (**B**; a and b), as a general marker for all fibroblast cells, was not significantly altered when comparing the $T\beta RII^{(fl/fl);PY}$ and $T\beta RII^{(fl/fl);PY;WC}$ tumor tissues (relative pixel density was 10.2 +/- 0.9 SEM *versus* 14.0 +/- 2.1 SEM respectively). Smooth muscle actin (SMA) expression (**B**; c and d) was significantly increased in $T\beta RII^{(fl/fl);PY;WC}$ tumor tissues when compared to $T\beta RII^{(fl/fl);PY}$ controls (relative pixel density was 6.4 +/- 0.4 SEM *versus* 2.2 +/- 0.3 SEM respectively; $p<0.0001$). No significant difference was observed in von Willebrand Factor (vWF) staining (**C**; a and b) when comparing the $T\beta RII^{(fl/fl);PY}$ and $T\beta RII^{(fl/fl);PY;WC}$ tumor tissues (relative pixel density was 0.35 +/- 0.04 SEM *versus* 0.36 +/- 0.06 SEM respectively). CD31 staining was decreased in the $T\beta RII^{(fl/fl);PY;WC}$ tumor tissues (**C**; c and d) when compared to the $T\beta RII^{(fl/fl);PY}$ controls (relative pixel density was 5.5 +/- 0.5 SEM *versus* 9.5 +/- 0.8 SEM respectively; $p<0.0005$). Statistical significance for pixel density measurements was determined using un-paired t-tests; n=6 individual tumors for each genotype and 3 random fields analyzed per tissue section.

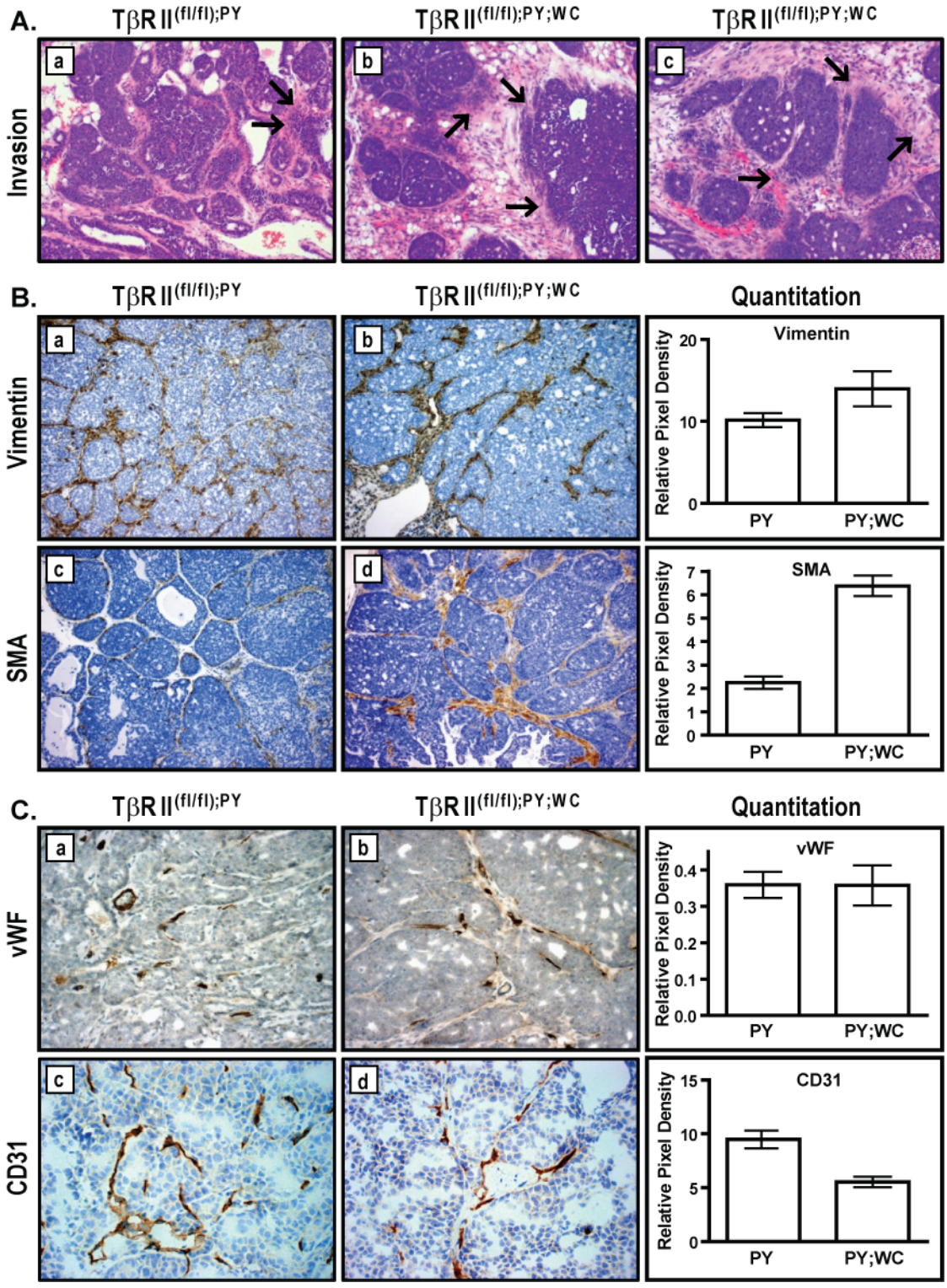


**Figure 16. TGF-β signaling within mammary carcinoma cell regulates the adjacent fibrovascular stroma.**

vWF is often associated with macrovasculature whereas CD31 is often associated with microvascular structures, and the two markers when analyzed together produce distinct complementary data related to tumor vascularization. We observed no difference in vWF abundance in the areas where this protein was detected (Figure 16C, a and b), however we did observe a significant decrease in the amount of CD31 expression in $T\beta RII^{(fl/fl);PY;WC}$ tumor tissues when compared with the $T\beta RII^{(fl/fl);PY}$ controls (Figure 16C, c and d). We subsequently analyzed the level of $VEGF^{165}$ mRNA expression by real-time PCR and found that there was not a significant difference in the level of expression when comparing the conditional TβRII null and control tumor tissues (data not shown). These results together suggested that the stromal expansion in $T\beta RII^{(fl/fl);PY;WC}$ tumor tissue was not simply the result of a general increase in angiogenesis.

**The loss of TβRII in mammary carcinoma cells can enhance recruitment of F4/80+ cells to the tumor microenvironment and increase the expression of pro-inflammatory genes including *Cxcl1*, *Cxcl5* and *Ptgs2* (COX-2)**

Recent work in our laboratory has demonstrated an abundant bone marrow derived inflammatory cell infiltrate often associated with areas of mammary tumor invasion (Yang et al., 2008). The morphology and tissue degradation surrounding areas of inflammation suggested that a major component of this infiltrate may include F4/80+ cells that have been previously implicated in the progression of human disease to malignancy (Leek et al., 1996). To analyze the abundance and localization of the F4/80+ cell populations within our tumor samples we performed immunohistochemistry (Figure 17A, a and b). We were able to detect a significant increase in the F4/80+ cell population

associated with our TβRII$^{(fl/fl);PY;WC}$ tumor tissues when compared to the TβRII$^{(fl/fl);PY}$ controls.

To determine the inflammatory factors regulated by TGF-β that may influence the recruitment of the F4/80+ cell population *in vivo*, it was necessary to first determine which inflammatory factors were produced by MMTV-PyVmT mammary carcinoma cells *in vitro*. We performed a cytokine and chemokine antibody array to identify factors that were produced and secreted by the carcinoma cells (Figure 17B). We were able to identify *Cxcl1*, *Cxcl5*, *Cxcl16*, *Ccl5*, *Ccl9* and *Ccl20* as chemotactic factors that were normally produced at a significant level by MMTV-PyVmT tumor cells. In order to further determine which factors were highly dependent on TGF-β signaling for their regulation we searched some of our preliminary microarray data. The microarray data set was produced to determine the changes in gene expression associated with TGF-β stimulation of control MMTV-PyVmT tumor cells for one hour *in vitro*. The results indicated that TGF-β decreased the expression of *Cxcl1*, *Cxcl5*, *Ccl20* and *Ptgs2* in the MMTV-PyVmT carcinoma cells (Figure 17C). The level of mRNA suppression was similar to c-myc, a well-known TGF-β repressed gene (Pietenpol et al., 1990). Further, when we compared the proteins expressed by the carcinoma cells to the genes regulated by TGF-β *in vitro*, the results suggested that deleting TβRII in the carcinoma epithelium would permit an increased level of *Cxcl1*, *Cxcl5* and *Ccl20* expression. We performed real-time PCR for *Cxcl1*, *Cxcl5*, *Cxcl16*, *Ccl5*, *Ccl9*, *Ccl20* and *Ptgs2* using RNA from our TβRII$^{(fl/fl);PY}$ control and TβRII$^{(fl/fl);PY;WC}$ tumor tissues to determine if the results obtained *in vitro* would be relevant *in vivo*.

**Figure 17. Inflammation and inflammatory gene expression.** TGF-β signaling regulates the infiltration of F4/80+ cells and correlates with the expression of genes known to promote inflammation including *Cxcl1*, *Cxcl5* and *Ptgs2* (COX-2) in primary mammary tumor tissues. **A.** F4/80+ bone marrow derived cell infiltration into primary $T\beta RII^{(fl/fl);PY}$ and $T\beta RII^{(fl/fl);PY;WC}$ tumor tissues. F4/80 IHC in $T\beta RII^{(fl/fl);PY}$ and $T\beta RII^{(fl/fl);PY;WC}$ tissues (a and b, respectively). The F4/80+ cell population (brown staining, arrows) was primarily localized in the stroma (S) along the leading edge between the distal pre-neoplastic hyperplasias and adjacent solid tumor tissues (T). Quantitation of the F4/80+ staining revealed a significant increase in F4/80+ cells recruited to the $T\beta RII^{(fl/fl);PY;WC}$ tumor microenvironment when compared with $T\beta RII^{(fl/fl);PY}$ controls ($p<0.001$). The $T\beta RII^{(fl/fl);PY;WC}$ tumors had a mean pixel density of 12.7 (+/- 1.7 SEM) whereas the $T\beta RII^{(fl/fl);PY}$ controls had a mean pixel density of 5.9 (+/- 0.6 SEM). Statistical significance was determined using an un-paired t-test with 6 individual tumors for each genotype and 3 random fields analyzed per tissue section. **B.** Identification of factors that were produced by $T\beta RII^{(fl/fl);PY}$ carcinoma cells using a cytokine antibody array incubated with conditioned medium that was collected after 24 hours of growth. Cytokine and chemokine proteins were captured on the antibody array membrane and visualized using a secondary chemoluminescent detection. Several chemotactic factors were detected at a relatively high level in the conditioned medium from $T\beta RII^{(fl/fl);PY}$ carcinoma cells including Cxcl1, Cxcl5, Cxcl16, Ccl5, Ccl9 and Ccl20. Background was determined using complete growth medium alone. **C.** Array data from $T\beta RII^{(fl/fl);PY}$ control cells *versus* $T\beta RII^{(fl/fl);PY}$ control cells +TGF-β (5ng/ml; 1 hour of stimulation) demonstrated expression changes in genes that are known to promote inflammation. In the presence of serum, TGF-β was able to suppress the expression of *Ccl20*, *Cxcl1*, *Cxcl5* and *Ptgs2* mRNA at a level that was similar to *c-myc* which is a gene known to be potently repressed by TGF-β signaling. **D.** Real-time PCR analysis of genes identified in B and C revealed that the loss of TGF-β signaling significantly increased the expression of *Cxcl1*, *Cxcl5* and *Ptgs2* in $T\beta RII^{(fl/fl);PY;WC}$ primary mammary tumor tissues when compared with the $T\beta RII^{(fl/fl);PY}$ controls ($p<0.0005$, $p<0.0001$ and $p<0.005$ respectively). No statistically significant difference was observed for *Cxcl16*, *Ccl5*, *Ccl9* or *Ccl20* in the primary mammary tumor tissues. Eight primary mammary tumor samples for each genotype (four from the fourth inguinal gland and four from the third thoracic gland) were used to produce RNA that was used for subsequent Real-time PCR experiments. Each tumor sample was tested in triplicate for each gene of interest and statistical significance was determined using un-paired t-tests for $(1/\Delta C_t)$ values normalized to 18S and transformed to the median.

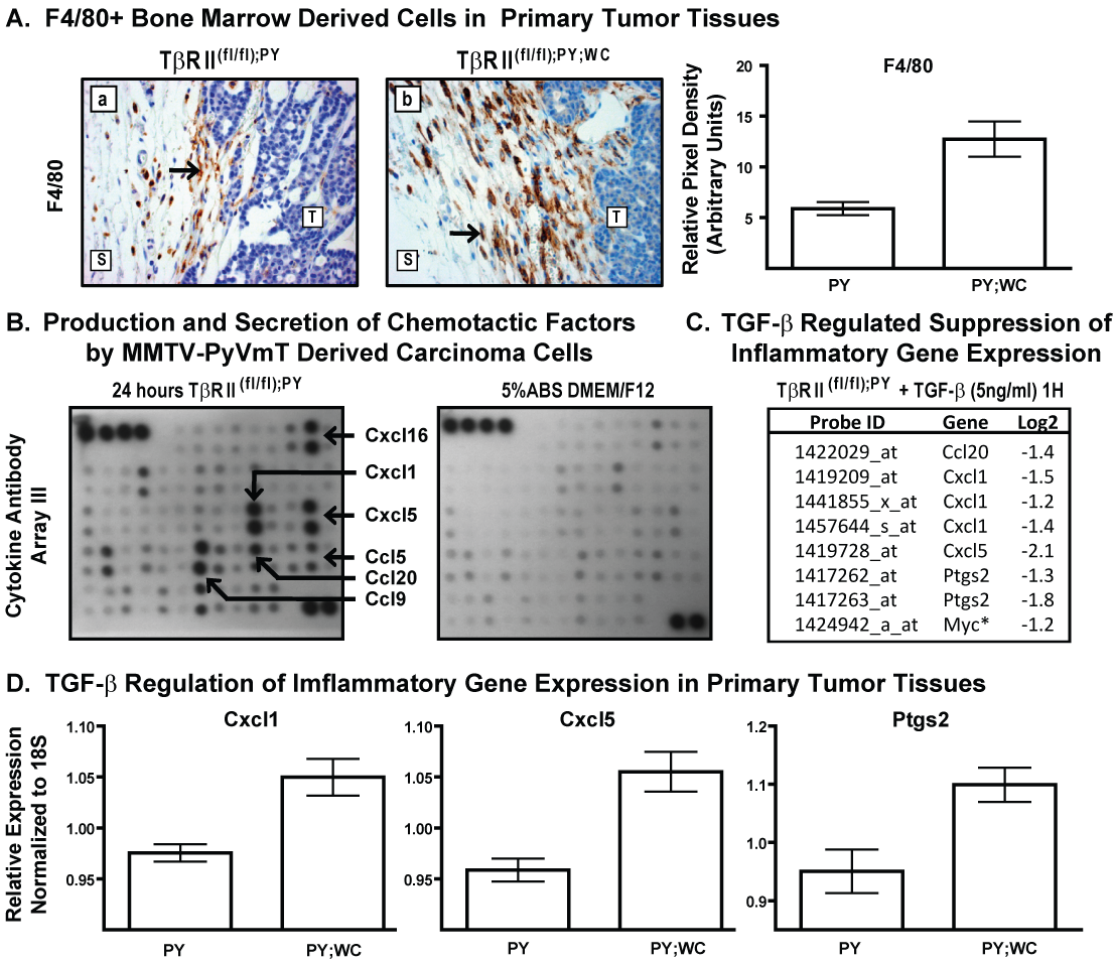


**Figure 17. Inflammation and inflammatory gene expression.**

The results indicated that *Cxcl1*, *Cxcl5* and *Ptgs2* mRNA was significantly upregulated in the TβRII$^{(fl/fl);PY;WC}$ tumor tissues when compared with the TβRII$^{(fl/fl);PY}$ controls (Figure 17D). Our results suggest that in mammary carcinoma cells, when TGF-β signaling is lost, the upregulation of pro-inflammatory factors can enhance the recruitment of bone marrow derived cell populations that are known to promote tumor progression and metastasis (Condeelis and Pollard, 2006; Coussens and Werb, 2002; Lewis and Pollard, 2006; Yang et al., 2008).

**Basal and myoepithelial cell markers cytokeratin 5 and p63 are more prevalent in mammary carcinomas that lack TβRII expression *in vivo***

Currently it is not known whether multipotent basal progenitors or lineage committed carcinoma cells in the primary mammary tumor microenvironment give rise to distant pulmonary metastases. Our initial observations in the primary tumors and pulmonary metastases suggested that moderate to well-differentiated carcinoma cells were responsible for the distant metastases. However, we wanted to address this issue more directly using previously defined mammary cell lineage markers since it is known that the MMTV promoter/enhancer (used to drive expression of the PyVmT oncogene) can express in all mammary epithelial cell lineages including the basal cell population. As a marker for the basal and myoepithelial cell populations we have examined cytokeratin 5 (K5) expression, which has been used previously to identify putative mammary progenitors, basal myoepithelium and bona fide basaloid carcinoma cells in situ (Abd El-Rehim et al., 2004; Mikaelian et al., 2006; van de Rijn et al., 2002).

**Figure 18. Keratin 5 and p63 localization in primary mammary tumors and lung metastases.** Loss of TGF-β signaling increased the abundance of carcinoma cell populations that express basal and myoepithelial markers in primary mammary tumors and associated lung metastases. Cytokeratin 5 (K5) expressing carcinoma cells were enriched in TβRII$^{(fl/fl);PY;WC}$ tumor tissues when compared to TβRII$^{(fl/fl);PY}$ controls (A and B). **A.** In the primary mammary tumors (a and b), K5 expression was localized in the carcinoma cell compartment adjacent to the fibrovascular stroma. K5 expressing cells constituted a minor sub-population of invasive cells (arrows) present within primary mammary carcinomas (c and d). **B.** K5 expressing cells were present in lung metastases associated with the MMTV-PyVmT tumor model. Abundant K5 expression was observed in all TβRII$^{(fl/fl);PY;WC}$ metastases (a and b). The large (a) and small (b) metastases present in the TβRII$^{(fl/fl);PY;WC}$ model expressed K5 in cells that surround the lobular alveolar structures associated with the moderate and well-differentiated lung metastases. K5 expression in the TβRII$^{(fl/fl);PY}$ model was more variable (c and d). In the large TβRII$^{(fl/fl);PY}$ lung metastases, K5 expression was often sparse or not observed in stained 5uM sections (c). In the small TβRII$^{(fl/fl);PY}$ lung metastases, K5 expression was variably expressed with some foci that had an abundant K5+ cell population surrounding moderate to well-differentiated lobular alveolar structures (d). **C.** Total p63 IHC staining of primary mammary tumor tissues. The distribution of p63 in the TβRII$^{(fl/fl);PY}$ and TβRII$^{(fl/fl);PY;WC}$ tumor tissues was similar to the K5 profile. Staining was predominantly observed around the outer edge of lobules adjacent to the fibrovascular stroma. **D.** Quantitation of the relative K5+ and p63+ cell populations within primary mammary tumor tissues. The relative pixel density for K5 was 33.4 (+/- 5.1 SEM) in the TβRII$^{(fl/fl);PY}$ tumor tissue *versus* 51.7 (+/- 4.6 SEM) in the TβRII$^{(fl/fl);PY;WC}$ model ($p<0.02$). The relative pixel density for total p63 was 46.5 (+/- 5.2 SEM) in the TβRII$^{(fl/fl);PY}$ tumor tissue *versus* 62.7 (+/- 4.0 SEM) in the TβRII$^{(fl/fl);PY;WC}$ model ($p<0.02$). Statistical significance for pixel density measurements was determined using un-paired t-tests; n=6 individual tumors for each genotype and 3 random fields analyzed per tissue section. (a and b) K5 and delta-Np63 (dNp63) IF demonstrating that all K5+ cells (green) were dNp63+ (red) whereas many dNp63+ cells were negative for K5 (arrows).

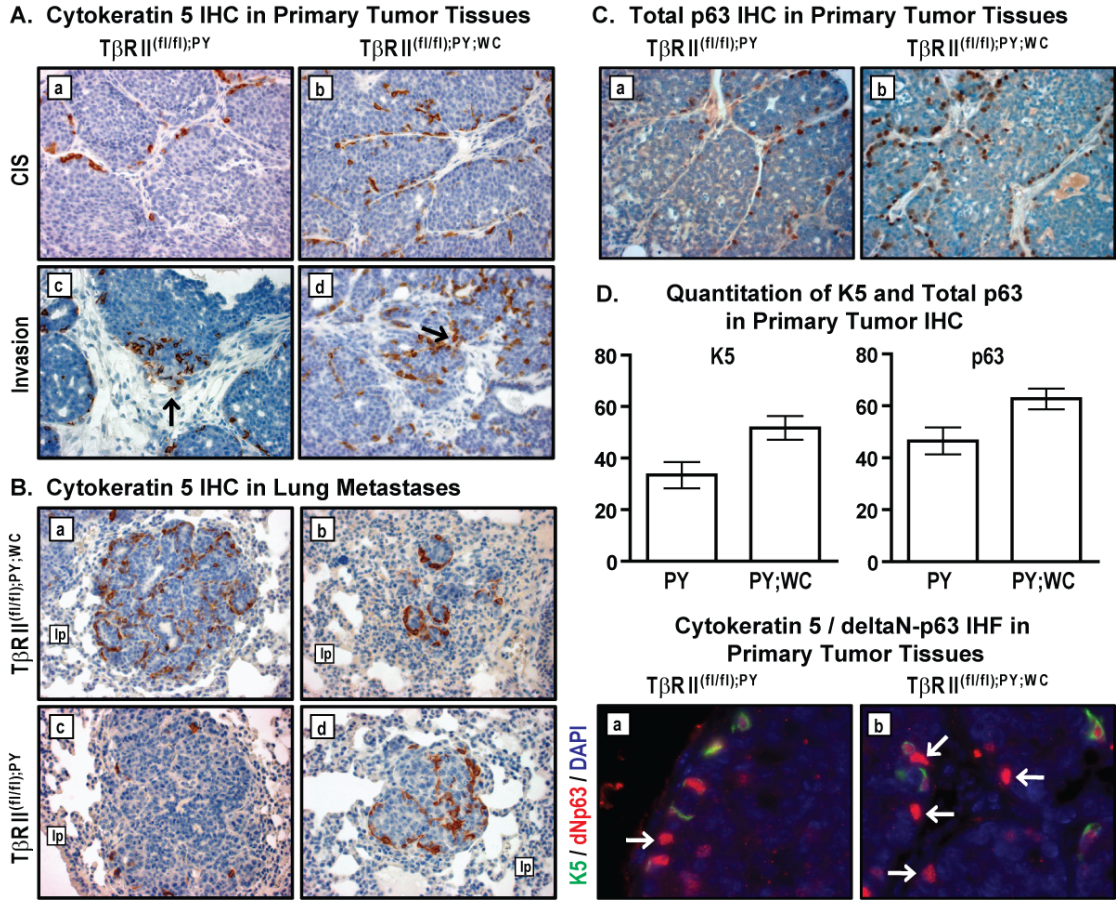


**Figure 18. Keratin 5 and p63 localization in primary mammary tumors and lung metastases.**

K5 expression was more abundant in the epithelial cell compartment of TβRII$^{(fl/fl);PY;WC}$ tumor tissues when compared with TβRII$^{(fl/fl);PY}$ controls (Figure 18A, a and b). In the TβRII$^{(fl/fl);PY}$ control tissues, K5 expression was predominantly limited to small lobules (Figure 19, a). However, the TβRII$^{(fl/fl);PY;WC}$ tumors had a relatively high number of K5+ cells throughout the tissue regardless of individual lobule size (Figure 19, b). The K5 staining, when present, in both tumor models predominantly localized at the outer edge of each lobule directly adjacent to the stroma (Figure 18A, a and b; Figure 19, a and b).

We observed invasive areas in both tumor models that contained K5+ cells (Figure 18A, c and d). Due to the moderate and well-differentiated lobular alveolar morphology of the lung metastases in both tumor models, we did not expect that K5+ cells would be present in the metastatic tumor tissues. However, we did entertain the possibility. To address this issue we performed IHC and IF for K5 on the TβRII$^{(fl/fl);PY}$ and TβRII$^{(fl/fl);PY;WC}$ lung metastases (Figure 18B, a-d). Interestingly, in the TβRII$^{(fl/fl);PY;WC}$ tumor model K5 expression was abundant in the lung metastases and localized in the same pattern observed in the primary lesions (Figure 18B, a and b). Every metastatic nodule identified in the TβRII$^{(fl/fl);PY;WC}$ model was associated with a prevalent K5+ cell population. In contrast, the TβRII$^{(fl/fl);PY}$ controls had fewer K5+ cells in the metastatic foci when present (Figure 18B, c and d) and in many cases the K5 positive cells were absent.

In order to determine if the K5+ population could be further stratified using additional basal and myoepithelial cell lineage markers, we performed IF co-localization with SMA and p63. Some of the K5+ cells were SMA+, however many of the K5+ cells

were SMA- (Figure 20A, a-d). The SMA+ cells likely represented differentiated myoepithelial cells whereas the SMA- population did not express this differentiation marker. p63 gene expression, like cytokeratin 5, has been associated with basal and myoepithelial cell populations (Barbareschi et al., 2001; Buono et al., 2006). We therefore performed immunohistochemistry for total p63 to determine if it was enriched to a similar extent as K5 (Figure 18C, a and b). The p63 stain had a similar spatial distribution as K5, however quantitation of the staining suggested that there were more p63+ cells than K5+ cells (Figure 18D, graphs). Due to the putative difference in K5 and total p63 abundance we performed IF to co-localize the two proteins in our tumor tissues. It is known that there are at least six alternate p63 isoforms, and we therefore limited our co-localization analyses to the deltaNp63 (dNp63) isoforms that have been previously associated with the early stages of progenitor cell differentiation (Figure 18D, a and b) (Barbareschi et al., 2001; Candi et al., 2007; Senoo et al., 2007). Every K5+ cell was also dNp63+. However, we also observed dNp63+ K5- cells in all primary and metastatic tumor tissues analyzed. These results suggested that the neoplastic K5+ dNp63+ cells represented a distinct MMTV-PyVmT carcinoma cell subpopulation. Next, we verified that the K5+ cells did not express markers indicative of genuine luminal cells. We performed immunofluorescence co-localization of K5 and cytokeratin 8 (K8), a known marker for the luminal cell lineage found within ducts and alveoli (Figure 20B, a-d) (Mikaelian et al., 2006). The K5+ cell population was absolutely distinct from the K8+ cell population, indicating that the K5 staining represented a subset of carcinoma cells rather than a trait acquired by partially differentiated carcinoma cells of ductal or lobular alveolar origin.

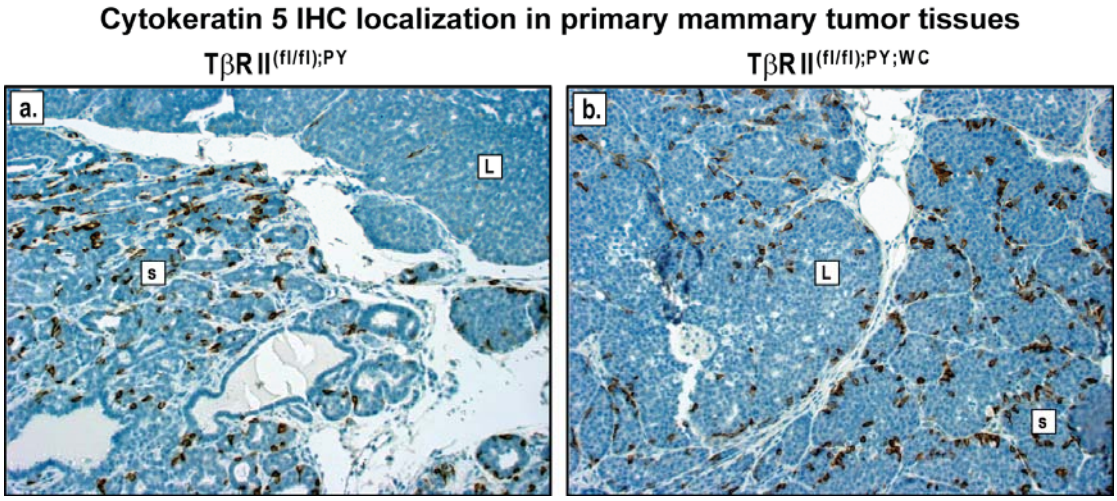


**Figure 19. Keratin 5 (K5) IHC localization in primary mammary tumor tissues.** K5 staining was more prevalent in tumors associated with the TβRII$^{(fl/fl);PY;WC}$ model when compared to those in the TβRII$^{(fl/fl);PY}$ controls (a and b). Specifically, the most significant difference in K5+ cell abundance was observed in the large tumor lobules (L). Smaller tumor lobules (s) in both models demonstrated the presence of K5+ carcinoma cells, however in TβRII$^{(fl/fl);PY}$ tumor tissues the K5 abundance negatively correlated with increased lobule size.

**Figure 20. IF analyses to determine K5/SMA and K5/K8 co-localization in primary mammary tumor tissues.** K5+ SMA+ cells were detected in the $T\beta RII^{(fl/fl);PY}$ and $T\beta RII^{(fl/fl);PY;WC}$ tumor models (data not shown). However, many K5+ SMA- cells were also detected in both tumor models (A, a-d; red). SMA+ stromal fibroblasts were included in each image as an internal control (A, a-d; green). K8 and K5 positive cells (B, a-d; red and green respectively) were mutually exclusive in all tumor tissues analyzed. DAPI was used to visualize cell nuclei (A, c and d; B, c and d; blue).

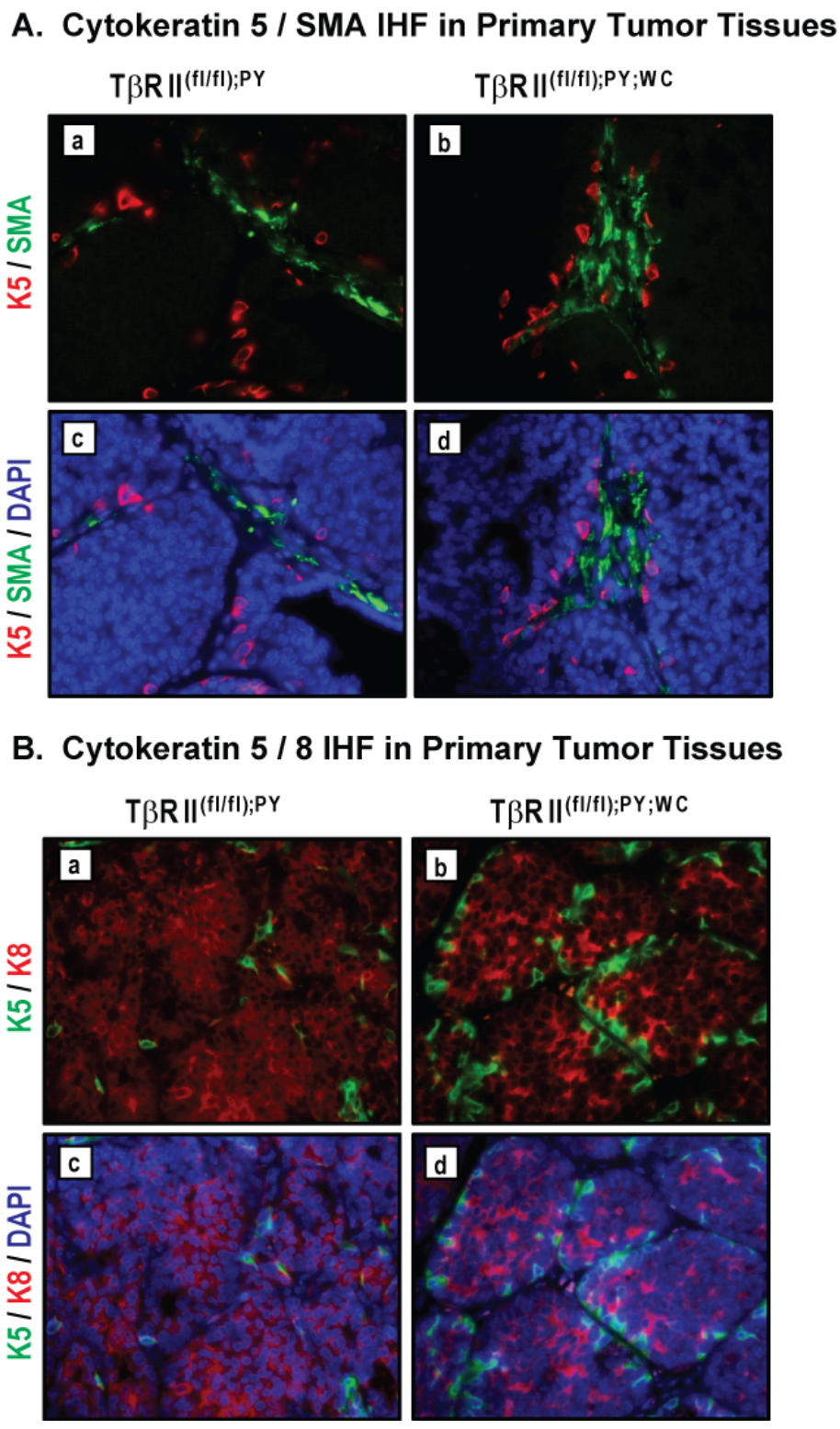


**Figure 20. IF analyses to determine K5/SMA and K5/K8 co-localization in primary mammary tumor tissues.**

## Discussion

TGF-β in normal epithelium is known to induce arrest of the cell cycle in G1, and during early tumor progression it has been suggested that this cytostatic regulation is a major contribution to carcinoma cell autonomous TGF-β mediated tumor suppression (Siegel and Massague, 2003). However, our data now suggests that the programmed cell death response to TGF-β signaling also plays a significant role in early mammary tumor suppression. The decrease in both typical and atypical PARP-1 cleavage products associated with the $T\beta RII^{(fl/fl);PY;WC}$ tumor tissue suggests that both the extrinsic and intrinsic caspase dependent and independent cell death pathways are impaired in the absence of TGF-β signaling (Bey et al., 2007). The inhibition of programmed cell death is an important consideration with regard to clinical treatment of cancer involving radiation or chemotherapy (Fulda and Debatin, 2006; Pommier et al., 2004). Radiation or conventional chemotherapies are often used to eliminate cancer cells that have been left behind during surgery, those that remain in circulation at the time of surgical resection or those that have already metastasized. Our current results suggest, that radiation and chemotherapies designed to induce carcinoma cell death may be less effective in eliminating the cells that have diminished TGF-β signaling during tumor progression. We are currently testing this hypothesis *in vitro* and *in vivo*.

It is now well known that carcinoma-associated fibroblasts (CAFs) can contribute to tumor progression (Barcellos-Hoff and Ravani, 2000; Hayward et al., 2001; Sakakura et al., 1981). However, it is not known specifically which endogenous fibroblast subpopulations are involved in the regulation of adjacent carcinoma progression. Previously, it has been shown that individual fibroblast populations can be differentially

classified based on their unique molecular signatures (Chang et al., 2002). The unique signatures obtained by mRNA expression profiling, suggested that much like epithelial or myeloid cells, there may be distinct fibroblast subpopulations present within each tissue type. Further, a recent study that used the vimentin, type I collagen, FSP (S100A4), αSMA, PDGFRβ and NG2 markers to examine fibroblast heterogeneity within mammary and pancreatic carcinomas indicated that several distinct fibroblast sub-populations could be identified and quantified within the tumor microenvironment (Sugimoto et al., 2006). Together these results provide evidence for a fibroblast contribution to tumor initiation and progression while suggesting that individual sub-populations of fibroblasts may play similar or alternate roles that together contribute to the regulation of tumor progression.

In our system, the difference in total stromal abundance, SMA expression and macro/microvascular phenotype suggests that TGF-β signaling within the carcinoma cell significantly regulates the composition of adjacent fibrovascular stroma in the mammary tumor microenvironment. Currently we do not know what factors the SMA+ fibroblast-like cells are producing or how these unknown factors impact tumor progression. It is known, however that myofibroblasts are often associated with the leading edge of invasive tumors and it has been suggested that they promote tumor progression (Bierie and Moses, 2006a). In our study, we observed SMA+ staining in the stroma associated with most of the invasive areas (data not shown), and this further suggests that a SMA+ tumor reactive stroma may be involved in early invasion thereby promoting progression to metastasis.

Chemokines are a group of proteins that potently promote tumor inflammation through recruiting host cells to the organ where they are expressed. The data generated in

this study suggests that *in vivo*, carcinoma cell specific loss of TGF-β signaling increases *Cxcl1*, *Cxcl5* and *Ptgs2* (COX-2) gene expression that correlates with increased infiltration of F4/80+ bone marrow derived cells to the tumor microenvironment. Further, we have shown that the TGF-β dependent chemokine expression observed in the tumor microenvironment likely involves direct tumor cell autonomous regulation of gene expression by TGF-β, though experiments demonstrating suppression of *Cxcl1*, *Cxcl5* and *Ptgs2* when carcinoma cells were treated with TGF-β1 *in vitro*.

In the normal mammary gland, a common progenitor cell can differentiate to produce lobular alveolar and ductal epithelium in addition to basal myoepithelium (Hennighausen and Robinson, 2005). Each of these cells types express different subsets of proteins that are often used to identify their lineage (Buono et al., 2006; Mikaelian et al., 2006; Shillingford et al., 2003). The basal and myoepithelial cell populations in mammary tissue are known to express cytokeratin 5. Importantly, cytokeratin 5 gene expression significantly correlates with a basal cell subtype classification that is known to have a poor prognosis in human breast cancer (Perou et al., 2000; Sorlie et al., 2003). In a study composed of 611 human breast cancer samples, cytokeratin 5/6 protein expression in node negative breast tumor tissue was a prognostic factor for poor clinical outcome, independent of primary tumor size or grade (van de Rijn et al., 2002). In a similar study composed of 1944 human breast cancer samples, cytokeratin 5/6 protein expression was correlated with poor prognosis in addition to loss of ER expression and early age of tumor onset (Abd El-Rehim et al., 2004).

The tumors produced in our mouse models were predominantly adenocarcinoma, however the data indicate that the loss of TGF-β signaling in mammary tumor precursors

can enrich for a K5+ cell population. The presence of K5+ cells did not classify the total tumor tissue as a basaloid subtype, however it did indicate that there was an increased number of carcinoma cells with basaloid characteristics in tissues lacking TβRII expression. Many of the K5+ cells in our tumor tissues were negative for SMA suggesting that they were less differentiated than the cells expressing SMA. In addition, all of the K5+ cells were dNp63+ and negative for the luminal epithelial cell marker K8. Together the SMA, K8 and dNp63 co-localization data suggest that the K5+ carcinoma cell population includes a poorly differentiated sub-population of cells that may contribute to tumor progression and metastasis in the absence of TβRII signaling *in vivo*. Further, our analyses revealed that the K5+ p63+ cell population was enriched within the corresponding pulmonary metastases. It is our current hypothesis that some of the K5+ cells are carcinoma progenitors that can metastasize then divide asymmetrically, resulting in progenitor expansion and amplification of differentiated progeny. In subsequent experiments, it would be informative to determine if the K5+ cells express additional markers such as CD44 or CD24 that will permit sorting to test if they can function as self-renewing carcinoma progenitors.

Our current results indicate that when TGF-β signaling is lost in the mammary tumor microenvironment, several factors should be considered including the impact on carcinoma cell apoptosis, regulation of adjacent stromal fibrovascular cell populations, carcinoma cell lineage selection, regulation of inflammatory gene expression, and infiltration of tumor promoting bone marrow derived cell populations to the tumor microenvironment. It is likely that together, these factors significantly contribute to the TGF-β mediated regulation of early tumor progression and metastasis.

# CHAPTER IV

# TGF-β DEPENDENT GENE EXPRESSION AND PATIENT PROGNOSIS

## Introduction

Transforming growth factor beta (TGF-β) ligands are known to significantly regulate tumor initiation, progression and metastasis. In human breast cancer, alterations in the carcinoma cell response to TGF-β signaling have been linked to tumor progression. It has been shown, using tissues from women with mammary epithelial hyperplasia lacking atypia, that decreased immunohistochemical staining for the type II TGF-β receptor (TβRII) correlated with an increased risk of developing invasive breast cancer (Gobbi et al., 1999). In this study, a three-fold reduction in the number of TβRII positive carcinoma cells correlated with an approximate three-fold increase in the risk of developing a subsequent invasive breast cancer (Gobbi et al., 1999). The loss of TβRII expression has also been correlated with high grade human *in situ* and invasive breast cancer (Gobbi et al., 2000). In addition, these observations fit previously reported data that demonstrated human breast cancer cells with deficient TβRII expression were more tumorigenic than the same tumor cells in which the receptor was experimentally re-introduced (Sun et al., 1994). Recently, it has been shown that silencing of the *TGFBR2* gene can occur through promoter hypermethylation in human breast carcinoma cells (Shipitsin et al., 2007). It has also been shown that in human breast cancer, the presence of a T29->C polymorphism in the *TGFB1* gene increased the serum levels of TGFβ1

ligand and correlated with a reduced risk of developing breast cancer (Ziv et al., 2001). However, the effect of TGF-β signaling is known to be context specific; TGF-β is thought to be an early tumor suppressor and late tumor promoter during disease progression. Recently, gene expression profiling was used to identify a signature in established human cell lines indicative of TGF-β stimulation (Padua et al., 2008). In this study, the TGF-β responsive gene expression signature was shown to predict for increased lung metastasis in human breast cancer whereas no significant correlation was made with metastasis to bone. Further, the correlation between TGF-β signaling and increased lung metastasis was more significant in association with estrogen receptor (ER) negative tumors. Interestingly, this effect was functionally validated using late stage breast cancer cells originally derived from a pleural effusion, wherein it was shown that TGF-β signaling primed the cells for lung metastasis through upregulation of *Angiopoietin-like 4* (*ANGPTL4*) gene expression (Padua et al., 2008).

To experimentally determine the impact of TGF-β signaling on tumor initiation, progression and metastasis *in vivo*, we and others have used several mouse models of mammary tumorigenesis with engineered alterations in TGF-β pathway signaling components. Using this approach, it was shown that TGF-β signaling could suppress early tumor progression. This was first demonstrated using a model that expressed TGFβ1 under control of the mouse mammary tumor virus enhancer/promoter (MMTV-Tgfβ1) (Pierce et al., 1995). In this study, it was shown that TGF-β conferred resistance to DMBA induced carcinogenesis and prolonged the latency of MMTV-TGFα (transforming growth factor alpha) induced mammary tumors. In transgenic mice that expressed TGF-β1 under control of the *whey acidic protein* promoter (WAP-Tgfβ1), it

was subsequently shown that TGF-β signaling was able to confer resistance to mammary tumors induced by injection of the Mouse mammary tumor virus (Boulanger and Smith, 2001).

These early observations were later paralleled by results obtained through expression of a dominant negative type II TGF-β receptor in mammary epithelium (MMTV-DNIIR) (Gorska et al., 2003). In this study, it was shown that attenuation of TGF-β signaling significantly decreased tumor latency in the MMTV-TGFα mouse model. However, expression of the MMTV-DNIIR transgene also resulted in decreased carcinoma cell invasion (Gorska et al., 2003). This correlated with previous results demonstrating that systemic inhibition of TGF-β signaling, through administration of an Fc conjugated TβRII (Fc:TβRII), resulted in resistance to spontaneous metastasis in the MMTV-Neu mouse mammary tumor model and an experimental metastasis model *via* carcinoma cell tail vein injection (Yang et al., 2002b). Several subsequent studies further illustrated the link between enhanced TGF-β signaling and enhanced metastasis *in vivo* (Muraoka-Cook et al., 2004; Muraoka-Cook et al., 2006; Muraoka et al., 2003; Siegel et al., 2003). Together the results suggested that secretion of TGF-β and thereby stimulation of all cells in the mammary tumor microenvironment could result in enhanced tumor metastasis while the effect on tumor growth was context dependent. Further, the data suggested that at least some of the invasion and metastasis effects were dependent upon direct carcinoma cell specific responses to TGF-β stimulation *in vivo*. However, it has now been shown in the MMTV-PyVmT (polyoma virus middle T antigen) model of mammary tumorigenesis, that there may be a significant difference between attenuation and complete abrogation of TGF-β signaling with regard to the regulation of metastasis

(Forrester et al., 2005). This study was the first to clearly demonstrate *in vivo*, that a complete loss of TGF-β response in mammary carcinoma cells could significantly increased the occurrence of spontaneous pulmonary metastases (Forrester et al., 2005).

In recent studies, we have been able to further validate the increased pulmonary metastasis when carcinoma cell specific TGF-β signaling is ablated (Bierie et al., 2008; Yang et al., 2008) and Chapter III. In addition, we have identified a decrease in apoptosis, increased abundance of adjacent smooth muscle actin positive fibrovasculature stroma, increased carcinoma cell heterogeneity, and increased inflammatory gene expression that correlated with bone marrow derived cell infiltration in TβRII ablated mammary carcinoma tissues when compared to the controls (Bierie et al., 2008; Yang et al., 2008) and Chapter III. However, it was difficult to understand precisely how TGF-β regulated these effects due to the complex nature of our observed compound phenotypic differences. Therefore, to address the mechanistic role for TGF-β signaling associated with regulation of tumorigenesis in this context, we have now isolated and established multiple parallel independent polyclonal carcinoma cell lines from control and TβRII ablated MMTV-PyVmT derived tumor models ($T\beta RII^{(fl/fl;PY)}$ and $T\beta RII^{(WKO;PY)}$ respectively) (Bierie et al., 2008) and Chapter III. Using these cell lines, we performed microarray analyses to determine the differences in gene expression between the models and in response to TGF-β stimulation. These analyses were conducted in an effort to identify mechanisms that mediate TGF-β dependent regulation of tumorigenesis *in vivo*, and the results indicate that a major function for TGF-β signaling is the regulation of chemokine expression.

The strength of functional analyses associated with mouse modeling is balanced by limitations in interpretation of individual observations with regard to the direct impact on human disease. However, molecular profiling offers an ability to directly assess the prognostic value of results obtained from genome scale networking interactions identified in experimental model systems. The power associated with molecular profiling in differentiating tumor subtypes and patient prognosis has been highlighted in recent years (Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003). We have been able to obtain high quality microarray profiles for 1646 human breast cancer tissues with well documented clinical data related to tumor size, lymph node involvement, estrogen receptor (ER) status, treatment regimen and time of relapse detection over a ten year period if present (Gene Expression Omnibus ID: GSE10886, GSE4922, GSE6532, GSE2845 and GSE2034)(Carroll et al., 2006; Ivshina et al., 2006; Loi et al., 2007; Loi et al., 2008; van 't Veer et al., 2002; van de Vijver et al., 2002; Wang et al., 2005b). Using the clinical data and gene profiles represented by these five data sets, we have been able to determine that the gene expression signature associated with our TβRII deficient carcinoma cells predicted an increased risk of early tumor recurrence during human breast cancer progression. Further, the patients presenting with Luminal A or ER+ subtype breast cancer and those in the hormone only treatment group demonstrated a significant elevation in the risk of recurrence if they had a gene expression profile that was similar to the TβRII deficient carcinoma cell signature at the time of tissue collection.

## Experimental Procedures

### Cell line derivation and culturing conditions

Carcinoma cell lines were derived from TβRII$^{(WKO;PY)}$ and TβRII$^{(fl/fl;PY)}$ primary tumors as previously described. Once established, cells were isolated then cultured in DMEM/F12 with 5% adult bovine serum as previously described (Bierie et al., 2008) and Chapter III. NMuMG cells were cultured in DMEM with 10% fetal calf serum and 150ul of insulin per 500ml of medium. HC11 cells were HC11 cells were a gift from Jeffery Rosen (Baylor College of Medicine, Houston) on behalf of Dr. Bernd Groner (Georg-Speyer-Haus, Institute for Biomedical Research, Frankfurt am Main, Germany) and were grown in RPMI 1640 medium containing 10% bovine calf serum, 5mg/ml insulin (Sigma), 10 ng/ml epidermal growth factor (Sigma), 2mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. Medium was changed twenty-three hours before collection for microarray and real-time PCR analyses. One hour before collection, the medium was aspirated and complete medium or complete medium containing TGF-β was added to the cells. Cell lines were approximately 70% confluent at the time of RNA isolation. Chemokine expression studies were carried out in the presence or absence of 10ng/ml TGF-β or 100ng/ml OSM that was added one hour prior to RNA isolation as indicated.

**Southern blot and TGF-β response analyses**

DNA and Southern blot analyses were performed as previously described. Briefly, DNA was collected using phenol-chloroform extraction and stored at 4°C until use. Southern blots were performed using 10ug of DNA after digestion with *BglII*. Blots were pre-hybridized and hybridized using a buffer containing 4X SSCP, 1X Denhardt's solution, 1% SDS and 100ug/ml of sheared salmon sperm. The previously described 3' probe for recombination (Chytil et al., 2002), was hybridized overnight at 65°C. All washes were also performed at 65°C. Tritiated thymidine incorporation was performed using $4x10^4$ cells that were plated in a 24-well dish and allowed to grow overnight. The next day, when cells were approximately 50% confluent, the medium was aspirated and replaced with complete medium or complete medium containing 0.1, 1.0 or 10.0ng/ml TGF-β. Twenty two hours later cells were pulsed with 3μCi of tritiated thymidine per well (Perkin Elmer Life Sciences, Boston, MA, USA). After two hours, the cells were fixed with 1 ml 10% trichloroacetic acid for 30 min at room temperature, followed by two additional 30 minute washes with 10% trichloroacetic acid. DNA was solubilized by incubation in 300 μl 0.2 N NaOH for 30 minutes. Radioactivity was counted using 100 μl of solubilized DNA in 4 ml of scintillation fluid. Six replicates were performed for thymidine incorporation analyses. Results were reported as the mean +/- standard error of the mean (SEM) and two-tailed un-paired t-tests were used to determine significance.

**H&E staining and immunofluorescence**

Hematoxylin and eosin staining was performed using standard techniques. Immunofluorescence was performed using standard techniques. Briefly, medium was aspirated and cells were washed with PBS then fixed in 10% neutral buffered formalin for 30 minutes at room temperature (RT). Cells were washed (2X) with PBS + 10mM glycine then permeabilized PBS + 0.2% Triton X-100 for 5 minutes at RT. Slides were washed (2X) with PBS + 10mM glycine. Slides were blocked with PBS containing 3% milk for 10 minutes at RT. Primary antibody (diluted in PBS + 3% milk) was added for 30 min at RT. Texas Red conjugated Phalloidin (Invitrogen, T7471) was used at 1:200 diluted in PBS + 3% milk. Slides were washed (3X) for 5 minutes in PBS and blocked for 3 minutes in 3% milk. Secondary antibodies were added at 1:800 for 30 minutes in the dark then washed (3x) for 5 minutes with PBS. Slides were mounted using ProLong Antifade Gold with DAPI. Texas Red conjugated phalloidin images were displayed in the green channel for the purpose of illustration.

**Hybrid Trizol and Rneasy RNA isolation**

Cells were grown and treated in T-75 flasks prior to collection. 1ml of Trizol per T-75 flask was used to lyse the cells. The lysates were incubated at room temperature for 5 minutes then 0.2ml of chloroform was added. The samples were shaken vigorously by hand for 15 seconds, incubated at room temperature for three minutes and centrifuged at 13,000g for 15 minutes at 4°C. 300ul from the aqueous phase was removed and an equal volume of 70% RNA-free EtOH was added slowly while mixing with a pipette tip to

avoid localized precipitation. The samples were loaded into an RNeasy column (Qiagen) and centrifuged for 30 seconds at 8,000g. 700µl buffer RW1 was added to the column and centrifuged for 30 seconds at 8,000g. 500µl of RPE buffer was added to the column and centrifuged for 30 sec at 8,000xg (2X; column was placed in a new collection tube for the second wash). The column was centrifuged for 1 min at 8,000g to eliminate the remaining buffer. RNA was eluted in a fresh RNase-free microfuge tube using 30µl of RNase-free water then stored at -80°C until use.

**Affymetrix microarray, real-time PCR and cytokine antibody array analyses**

Samples were checked for integrity and subjected to microarray hybridization using the Affymetrix Mouse 430 2.0 gene chip according to standard protocols (available from the Vanderbilt Microarray Shared Resource core upon request). Genes were selected if they met all of the following criteria: signal was up- or downregulated at least 1.5 fold in all experimental samples when compared to the controls, at least two of the three experimental samples represented a 2.0 fold or higher change in expression when compared to the controls, and the CV value for the control group was less than 2.0. Validation for microarray data was conducted using real-time PCR according to the manufacturer's instructions with a custom template and proprietary primer sets for the reported gene products (SuperArray, custom template #CAPM-0439). Genes included for validation from the $T\beta RII^{(WKO;PY)}$ and TGF-β treatment signatures were analyzed using $T\beta RII^{(WKO;PY)}$, $T\beta RII^{(WKO;PY)}$ + TGF-β (10ng/ml), $T\beta RII^{(fl/fl;PY)}$, $T\beta RII^{(fl/fl;PY)}$ + TGF-β (10ng/ml) samples. In grouped analyses, results with a two-tailed unpaired t-test

$p<0.05$ were reported as significant. In paired analyses, results were reported if the fold change moved in the same direction for all samples and was at least 1.5 in all three samples with at least a 2.0 or greater fold change in the other two samples. *Cxcl1*, *Cxcl5* and *Ccl20* expression analyses in HC11 and NMuMG cells were conducted using the primer sets and conditions previously reported (Bierie et al., 2008) and Chapter III. Results were reported as mean of the median transformed $1/\Delta Ct$ values +/- standard error of the mean (SEM) and two-tailed un-paired t-tests were used to determine significance. The cytokine antibody array was performed using conditioned medium as previously described (Bierie et al., 2008) and Chapter III. Results were reported as the mean +/- standard error of the mean (SEM) and two-tailed un-paired t-tests were used to determine significance. Correlative analyses for our gene expression signatures with human gene profiling and clinical status data, were performed using normalized data representing 1646 patients from five independent previously reported studies (Gene Expression Omnibus ID: GSE10886, GSE4922, GSE6532, GSE2845 and GSE2034)(Carroll et al., 2006; Ivshina et al., 2006; Loi et al., 2007; Loi et al., 2008; van 't Veer et al., 2002; van de Vijver et al., 2002; Wang et al., 2005b). Probes were median centered across each dataset to minimize platform effects. Gene symbols were assigned using the manufacturer provided annotation, and duplicate gene symbols were collapsed by averaging within each sample. Breast cancer subtypes were assigned based on a nearest centroid classifier (Parker et al., submitted). Analysis of variance (ANOVA) was used to test the null hypothesis that the mean correlation to a signature is equal across the breast cancer subtypes. The similarity of each human breast cancer test case to the $T\beta RII^{(WKO;PY)}$ or TGF-β treated $T\beta RII^{(fl/fl;PY)}$ carcinoma cell signatures was computed using Pearson

correlation. Correlations were associated with relapse-free survival using univariate Cox proportional hazards. Categorical survival analyses were performed by splitting samples into groups based on the positive or negative correlation with the signature. Association of these groups with relapse-free survival was evaluated with the Log Rank test and visualized with Kaplan-Meier plots.

## Results

### PyVmT mammary carcinoma cells exhibit an epithelial to mesenchymal transition (EMT) in response to TGF-β stimulation

To determine functional and molecular roles for TGF-β signaling in mammary carcinoma cells we established individually derived polyclonal carcinoma cell lines from $T\beta RII^{(fl/fl;PY)}$ control and $T\beta RII^{(WKO;PY)}$ mammary tumors. Three of the cell lines from each model were selected for analysis. The cell lines were screened for TβRII recombination (Figure 21A). The $T\beta RII^{(fl/fl;PY)}$ control cells demonstrated presence of the floxed allele without any evidence of recombination. The $T\beta RII^{(WKO;PY)}$ cells were 100% recombined with no evidence of a loxP flanked TβRII allele. The recombination efficiency was functionally validated using a TGF-β growth response assay twenty-four hours after stimulation. The $T\beta RII^{(fl/fl;PY)}$ control cells demonstrated a dose dependent response to TGF-β stimulation that resulted in reduced tritiated thymidine incorporation (Figure 21A). As expected, the $T\beta RII^{(WKO;PY)}$ cells did not demonstrate a significant difference in tritiated thymidine incorporation after TGF-β treatment.

**Figure 21. Recombination of TβRII and induction of TGF-β dependent EMT in MMTV-PyVmT mammary carcinoma cells. A.** Southern blot analysis of the established polyclonal mammary carcinoma cell lines and respective TGF-β dependent growth responses *in vitro*. Analysis of southern blot hybridization demonstrated that the independently derived polyclonal TβRII$^{(fl/fl;PY)}$ control mammary carcinoma cells (FL1, FL2 and FL3; biological replicates) had intact floxed *Tgfbr2* alleles with no evidence of recombination. Alternatively, the independently derived polyclonal TβRII$^{(WKO;PY)}$ carcinoma cells (KO1, KO2 and KO3; biological replicates), were completely recombined with no evidence of a remaining floxed *Tgfbr2* allele. As expected, the growth of TβRII$^{(fl/fl;PY)}$ control carcinoma cell lines, measured by tritiated thymidine incorporation 24 hours after stimulation, was significantly inhibited by TGF-β stimulation whereas the TβRII$^{(WKO;PY)}$ carcinoma cell growth was not altered. **B.** In response to TGF-β stimulation (10ng/ml) for 48 hours, the TβRII$^{(fl/fl;PY)}$ control carcinoma cells demonstrated consistent changes in morphology and cell scattering that suggested an epithelial to mesenchymal transition had occurred. **C.** Loss of E-cadherin (a and b) and changes in F-actin localization from the cell membrane (c) to predominant association with stress fibers (d) further confirmed an EMT-like state in TβRII$^{(fl/fl;PY)}$ cells that had been treated with 10ng/ml of TGF-β for 48 hours. **D.** Loss of TGF-β signaling in TβRII$^{(WKO;PY)}$ cells did not result in a spontaneous state of EMT, as determined by the presence of membrane bound E-cadherin, in the presence or absence of TGF-β ligand at 10ng/ml after 48 hours of stimulation.

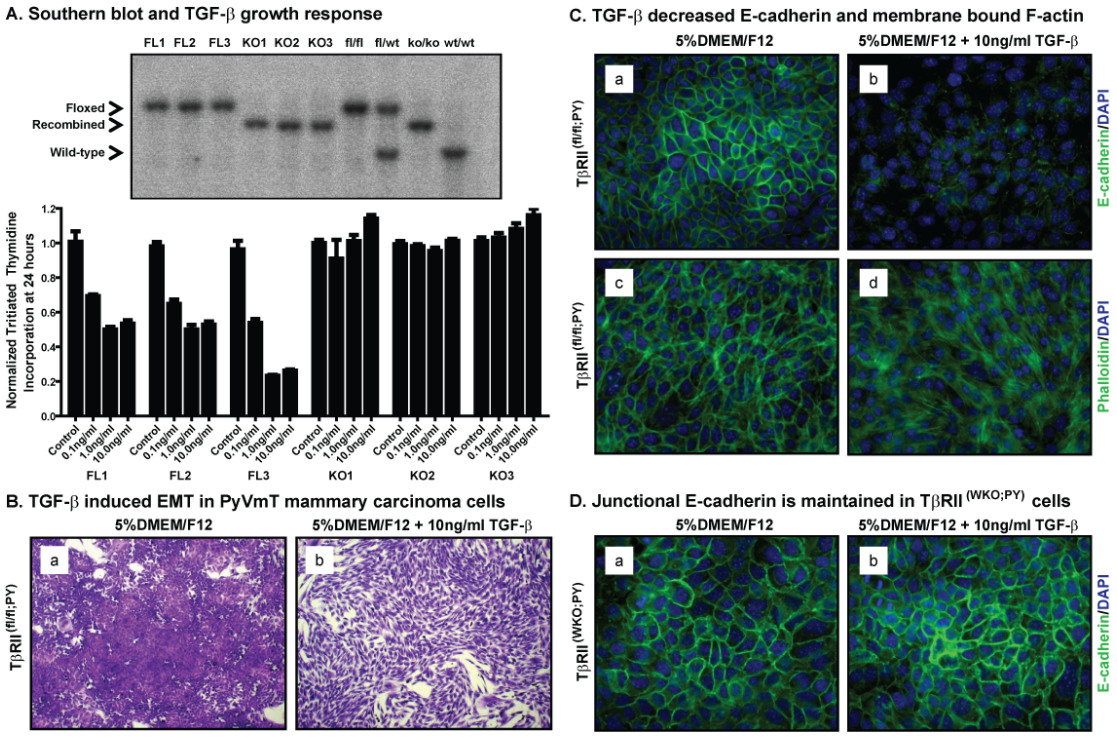


**Figure 21. Recombination of TβRII and induction of TGF-β dependent EMT in MMTV-PyVmT mammary carcinoma cells.**

It has recently been shown that some, but not all epithelial cell lines respond to TGF-β with an epithelial to mesenchymal transition (EMT) (Brown et al., 2004). The EMT process has been linked to increased carcinoma cell motility, invasion and metastasis (Oft et al., 2002; Oft et al., 1998; Onder et al., 2008; Yang et al., 2004a). *In vitro*, the control TβRII$^{(fl/fl;PY)}$ control lines were predominantly present in clusters of cells with an epithelial morphology (Figure 21B, a). In response to TGF-β, the TβRII$^{(fl/fl;PY)}$ control cell lines exhibited an elongated, fibroblast-like morphology and cell scattering that are hallmarks of EMT (Figure 21B, b). The changes in cell morphology correlated with loss of E-cadherin and F-actin tethering to the cell surface (Figure 21C and 21D). In TGF-β treated tissues, E-cadherin appeared to be degraded rather than accumulate in the cytoplasm (Figure 21C, b). In complete medium F-actin was detected in the cytoplasm and tethered to the cell membrane, however when TGF-β was added to the medium F-actin was predominantly associated with stress fibers in the cytoplasm (Figure 21C, d). Interestingly, N-cadherin was not significantly upregulated in the EMT-like carcinoma cells after the observed TGF-β dependent response (data not shown). Due to the enhanced rate of metastatic spread observed in our TβRII$^{(WKO;PY)}$ model, we also hypothesized that the mammary carcinoma cells lacking TβRII expression may have an increased sensitivity to growth factors or a predisposition toward spontaneous EMT. However, the response to serum was similar for the TβRII$^{(fl/fl;PY)}$ control and TβRII$^{(WKO;PY)}$ cell lines. In addition , the TβRII$^{(WKO;PY)}$ cells did not exhibit a spontaneous EMT under complete culturing conditions or when cultured in the presence of TGF-β (Figure 21D). Together, the data demonstrated that our TβRII$^{(fl/fl;PY)}$ control and TβRII$^{(WKO;PY)}$ cell lines were suitable for comparison. Further, differences in

response to growth factor stimulation or spontaneous EMT could not account for the enhanced metastatic spread observed in the TβRII$^{(WKO;PY)}$ model when compared with the TβRII$^{(fl/fl;PY)}$ controls.

**Molecular profiling revealed a difference in expression of genes that are known to regulate tumor progression**

To understand how TGF-β could regulate carcinoma cell associated gene expression, which ultimately regulates tumor progression and metastasis, we performed Affymetrix microarray analyses. TβRII$^{(WKO;PY)}$, TβRII$^{(fl/fl;PY)}$ and TGF-β treated TβRII$^{(fl/fl;PY)}$ cell lines were hybridized for subsequent analyses. We were able to identify 108 genes that were upregulated and 48 genes that were downregulated in TβRII$^{(WKO;PY)}$ cell lines when compared with the TβRII$^{(fl/fl;PY)}$ controls (Table 1). To determine the genes that were acutely regulated by TGF-β we performed pairwise analyses using TβRII$^{(fl/fl;PY)}$ mammary carcinoma cells in the presence or absence of TGF-β treatment for one hour prior to collection. Genes were selected if they were consistently differentially regulated in the same direction in all three cell lines. Using the paired analysis strategy, we were able to identify 41 genes that were consistently upregulated and 16 that were consistently downregulated in response to TGF-β treatment (Table 2). Real-time PCR validation and was performed using a common pool of genes selected from both experiments with TβRII$^{(WKO;PY)}$, TβRII$^{(fl/fl;PY)}$, TβRII$^{(WKO;PY)}$ + TGF-β and TβRII$^{(fl/fl;PY)}$ + TGF-β samples.

We were able to validate significant differences ($p<.05$) in the expression of 11 genes identified by microarray in the TβRII$^{(WKO;PY)}$, TβRII$^{(fl/fl;PY)}$ dataset using real-time

PCR analyses (Table 3, top; clustered in Figure 22A). In the list of validated genes validated by real-time PCR, one out of the 11 (*Junb*) was actually identified in the TGF-β treatment experiment but did not meet the filtering criteria for our $T\beta RII^{(WKO;PY)}$ *versus* $T\beta RII^{(WKO;PY)}$ microarray analyses. This suggested that our conservative filtering approach likely excluded some genes that were differentially regulated, however for the purpose of determining a gene expression signature this was preferable. TGF-β was present in the complete cell culture medium, however the amount was not enough to observe a difference in activation of downstream Smad pathways when comparing serum free to complete medium conditions. Therefore, to better model the stimulation likely experienced by the carcinoma cells during tumor progression, we also performed validation in the presence of TGF-β. When stimulated with TGF-β, we were able to detect a significant difference ($p<.05$) in the expression of 21 genes using the pooled gene validation approach (Table 3, bottom; clustered in Figure 22B). Importantly, we identified differences in expression of genes that are known to be TGF-β responsive including *Serpine1* (PAI-1), *Smad6*, *PDGF-β*, *Gadd45a*, *Ctgf*, *Lmcd1* and *Bcl2l11* (Bim). The genes identified as differentially expressed included some that offered potential mechanisms our previous observations *in vivo*. *Bim* and *Itgb3* have both been linked to promoting apoptosis and their expression was higher in the control cells. This is important, since we have observed higher rates of apoptosis in the control tumor tissues *in situ*. *Wisp1*, a CTGF family member, was also expressed at a higher level in the control cells and this gene has been genetically identified as a likely tumor suppressor.

**Table 1. Differentially expressed genes identified when TβRII$^{(WKO;PY)}$ and TβRII$^{(fl/fl;PY)}$ mammary carcinoma cells were compared.** Genes that had a higher level of expression in the TβRII$^{(WKO;PY)}$ samples were considered upregulated and those that were lower in the TβRII$^{(WKO;PY)}$ model were considered downregulated. Genes were selected if they met all of the following criteria: signal was consistently up- or downregulated at least 1.5 fold in all TβRII$^{(WKO;PY)}$ samples when compared to the TβRII$^{(fl/fl;PY)}$ controls, at least two of the three experimental samples represented a 2.0 fold or higher change in expression when compared to the TβRII$^{(fl/fl;PY)}$ controls, and the CV value for the TβRII$^{(fl/fl;PY)}$ group was less than 2.0.

**Table 1. Differentially expressed genes identified when TβRII$^{(WKO;PY)}$ and TβRII$^{(fl/fl;PY)}$ mammary carcinoma cells were compared.**

| Downregulated in TβRII$^{(WKO;PY)}$ | | | |
|---|---|---|---|
| Adamts3 | Crlf1 | Megf10 | Serpine1 |
| Adcy7 | Cxcl12 | Megf9 | Slc35f1 |
| Aldoc | Dpysl5 | Mtap9 | Sox5 |
| Alg13 | Elavl2 | Myo7a | Ssr1 |
| Alox5ap | Enpp3 | Pcdh21 | St8sia2 |
| Ankrd29 | F2r | Pde2a | Tbx15 |
| Apex2 | Gja7 | Pdgfb | Tmeff1 |
| Car2 | Gm22 | Plagl1 | Tmem29 |
| Car9 | Gna14 | Rgs10 | Tspan7 |
| Chst11 | Igf1 | Rspo3 | Tyms |
| Cnr1 | Il17b | Satb1 | Vim |
| Col9a3 | Itgb3 | Sbk1 | Wisp1 |

| Upregulated in TβRII$^{(WKO;PY)}$ | | | |
|---|---|---|---|
| Adar | Gbp6 | Lgals9 | Rsad2 |
| Angptl2 | Gjb4 | Lgr6 | Rtp4 |
| Ankrd1 | Gprc5c | Mlstd2 | S100a6 |
| Anxa1 | Gvin1 | Mt1 | Saa3 |
| B2m | H2-K1 | Mt2 | Scara5 |
| B3gnt3 | H2-Q1 | Mx1 | Sdc4 |
| Brd4 | H2-Q8 | Mx2 | Sectm1b |
| Btg2 | H2-T10 | Nmi | Sftpd |
| C1qdc2 | H2-T23 | Nr4a1 | Slfn2 |
| C1r | Hist1h1c | Nradd | Slpi |
| C79267 | Hist1h2bc | Nuak2 | Sp100 |
| Ccl20 | Hist3h2a | Oas1g | Stambpl1 |
| Ccl5 | Hspb8 | Oas2 | Stat1 |
| Clec2d | Id3 | Oas3 | Stat2 |
| Col4a6 | Ids | Oasl1 | Syce2 |
| Csprs | Ifi203 | Parp12 | Tanc2 |
| Ctsa | Ifi35 | Parp14 | Tap2 |
| Cxcl1 | Ifih1 | Parp3 | Tmem140 |
| Cxcl16 | Ifit3 | Parp8 | Tnfrsf1b |
| Cyp1b1 | Irf1 | Parp9 | Tns4 |
| D11Lgp2e | Irgm | Phf11 | Tor3a |
| Dleu2 | Isgf3g | Pim3 | Tpm2 |
| Drbp1 | Khdrbs3 | Pla2g7 | Trex1 |
| Egr1 | Krt17 | Plk2 | Tyki |
| Ehbp1l1 | Lamc2 | Plk3 | Ube1l |
| Eif2ak2 | Lgals3 | Psmb2 | Ube2l6 |
| Gbp2 | Lgals3bp | Psmb9 | Zbp1 |

**Table 2. Differentially expressed genes identified as a result of TGF-β stimulation in TβRII$^{(fl/fl;PY)}$ mammary carcinoma cells.** Genes that had a higher level of expression in response to TGF-β treatment were considered upregulated and those that were lower were considered downregulated. Genes were selected if they met the following criteria: signal was consistently up- or downregulated at least 1.5 fold in all TGF-β treated samples when compared to the controls, at least two of the three TGF-β treated samples represented a 2.0 fold or higher change in expression when compared to the controls.

**Downregulated by TGF-β in TβRII$^{(fl/fl;PY)}$**

| | | | |
|---|---|---|---|
| Adamts1 | Cdc42ep3 | Cxcl1 | Epgn |
| Adamts15 | Cebpd | Cxcl5 | Gdap10 |
| Alcam | Chka | Cyp1b1 | Ppp1r3c |
| Ccl20 | Csn3 | Dusp6 | Tslp |

**Upregulated by TGF-β in TβRII$^{(fl/fl;PY)}$**

| | | | |
|---|---|---|---|
| Adora1 | Edn2 | Lmcd1 | Sh3bp2 |
| Bcl11a | Egr2 | Lrig3 | Slc20a1 |
| Bcl2l11 | Egr3 | Lrp4 | Smad6 |
| Bhlhb2 | Fos | Map3k14 | Spsb1 |
| Camk2n1 | Fosb | Mfsd2 | Tmem98 |
| Ctgf | Foxq1 | Myo1d | Wisp1 |
| Ctla2a | Gadd45a | Pdgfb | Wnt9a |
| Ctla2b | Gadd45g | Plekhh1 | Zfp750 |
| Cxcl12 | Gja3 | Rasl11b | |
| Cxcr4 | Gse1 | Serpine1 | |
| Ddit4 | Junb | Sfn | |

**Table 3. Real-time PCR validation of differences in gene expression associated with TβRII$^{(WKO;PY)}$ and TβRII$^{(fl/fl;PY)}$ mammary carcinoma cells.** Real-time PCR was performed using the biological replicates for each model and the 1/ΔCt values were used to determine the significance using two-tailed, unpaired t-tests. Validation was performed using TβRII$^{(WKO;PY)}$ and TβRII$^{(fl/fl;PY)}$ mammary carcinoma cells cultured in complete medium (top). To further stratify the differences between the cell populations validation was also performed using TβRII$^{(WKO;PY)}$ cells cultured in complete medium compared with TβRII$^{(fl/fl;PY)}$ cells cultured in complete medium containing TGF-β ligand at 10ng/ml one hour after stimulation (bottom). No differences in gene expression were observed when TβRII$^{(WKO;PY)}$ cells cultured in complete medium were compared with TβRII$^{(WKO;PY)}$ cells cultured in complete medium containing TGF-β ligand at 10ng/ml one hour after stimulation. Values were normalized to *Gusb*, *Hprt1*, *Hsp90ab1*, *Actb* and *PPIA*.

**Table 3. Real-time PCR validation of differences in gene expression associated with TβRII$^{(WKO;PY)}$ and TβRII$^{(fl/fl;PY)}$ mammary carcinoma cells.**

Complete medium

| Gene Symbol | Fold Change | p-value |
|---|---|---|
| S100a6 | 2.6 | 0.04 |
| Ctsa | 2.4 | 0.03 |
| Bst2 | 2.0 | 0.01 |
| Junb | 1.7 | 0.03 |
| Pdgfb | -1.6 | 0.05 |
| Itgb3 | -1.9 | 0.02 |
| Tbx15 | -2.1 | 0.05 |
| Tspan7 | -2.5 | 0.01 |
| F2r | -2.8 | 0.03 |
| Pcdh21 | -3.6 | 0.003 |
| Vim | -22.3 | 0.03 |

Complete medium + TGF-β

| Gene Symbol | Fold Change | p-value |
|---|---|---|
| Cxcl1 | 14.8 | 0.04 |
| Cxcl5 | 7.6 | 0.03 |
| Bst2 | 2.8 | 0.02 |
| Ctsa | 2.0 | 0.03 |
| Bcl2l11 | -3.5 | 0.04 |
| Tspan7 | -3.9 | 0.03 |
| F2r | -4.1 | 0.008 |
| Itgb3 | -4.2 | 0.02 |
| Wnt9a | -4.2 | 0.03 |
| Pdgfb | -4.8 | 0.001 |
| Pcdh21 | -4.8 | 0.001 |
| Cxcl12 | -5.7 | 0.03 |
| Camk2n1 | -6.2 | 0.03 |
| Ctgf | -6.7 | 0.04 |
| Smad6 | -6.8 | 0.02 |
| Gadd45a | -7.3 | 0.004 |
| Cxcr4 | -8.0 | 0.03 |
| Wisp1 | -10.7 | 0.02 |
| Lmcd1 | -13.4 | 0.03 |
| Vim | -24.4 | 0.02 |
| Serpine1 | -62.5 | 0.01 |

**Figure 22. Cluster analyses for genes that were validated by real-time PCR.** Genes that demonstrated a significant difference in expression with a two-tailed unpaired t-test value of 0.05 or less were used for clustering. Each row represents the heatmap interpretation of 1/ΔCt values associated with a specific gene. **A.** $T\beta RII^{(WKO;PY)}$ and $T\beta RII^{(fl/fl;PY)}$ carcinoma cell lines cultured in complete medium. **B.** $T\beta RII^{(WKO;PY)}$ cells cultured in complete medium compared with $T\beta RII^{(fl/fl;PY)}$ carcinoma cells in the presence of complete medium with TGF-β at 10ng/ml one hour after stimulation. No differences in gene expression were observed when $T\beta RII^{(WKO;PY)}$ cells cultured in complete medium were compared with $T\beta RII^{(WKO;PY)}$ cells cultured in complete medium containing TGF-β ligand at 10ng/ml one hour after stimulation. FL1, FL2 and FL3, $T\beta RII^{(fl/fl;PY)}$; KO1, KO2 and KO3, $T\beta RII^{(WKO;PY)}$. Values were normalized to *Gusb*, *Hprt1*, *Hsp90ab1*, *Actb* and *PPIA*.

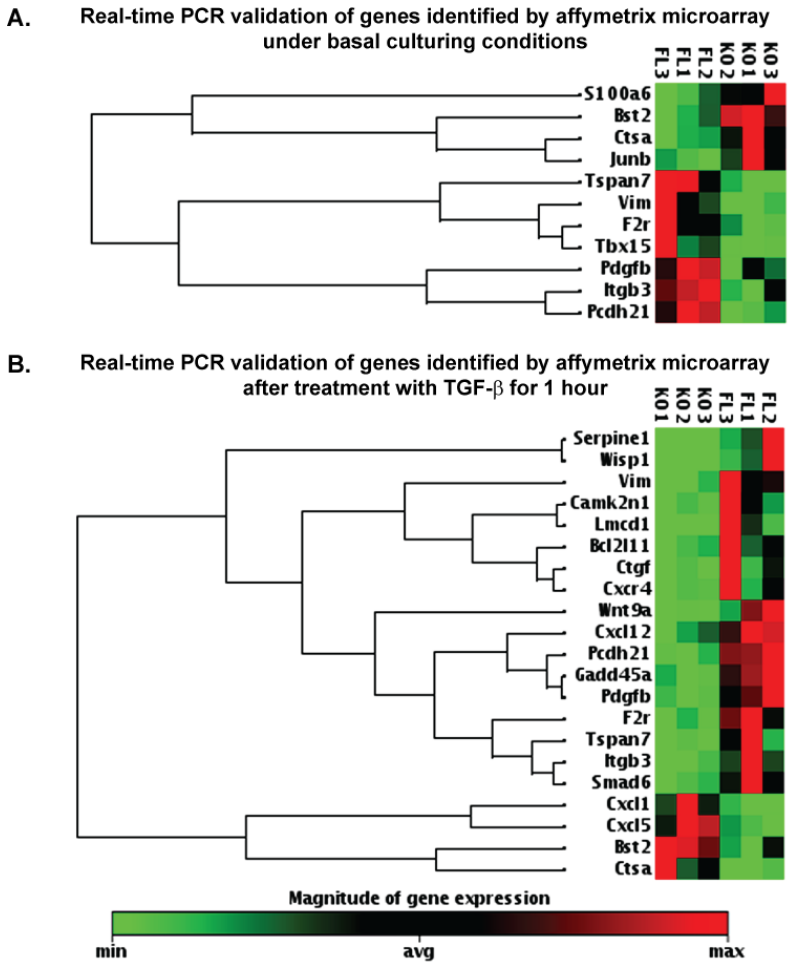


**Figure 22. Cluster analyses for genes that were validated by real-time PCR.**

**Figure 23. TGF-β dependent chemokine protein secretion by mammary carcinoma cells and the effect of Cxcl1 stimulation on metastatic mammary carcinoma cell migration. A.** Conditioned medium from $T\beta RII^{(WKO;PY)}$ and $T\beta RII^{(fl/fl;PY)}$ mammary carcinoma cells revealed increased secretion of Cxcl1 and Cxcl5 protein by the $T\beta RII^{(WKO;PY)}$ populations. Quantitation of Cxcl1 and Cxcl5 presence was performed and represented at the median transformed mean values +/- standard error of the mean (SEM). Two-tailed unpaired t-tests were performed and the values were significant with p-values<0.05. Ctl, $T\beta RII^{(fl/fl;PY)}$; KO, $T\beta RII^{(WKO;PY)}$. **B.** Wound closure assays were used to determine the effect of Cxcl1 presence on metastatic carcinoma cell migration. Values reported as mean percentage +/- (SEM). In the 4T1 carcinoma cell line, two-tailed unpaired t-test p-values were 0.5018 at 5ng/ml, 0.0853 at 20ng/ml, 0.0588 at 40ng/ml and less than 0.005 at 80ng/ml. In the MDA-MB-231 cell line, two-tailed unpaired t-test p-values were 0.0019 at 5ng/ml, 0.0411 at 20ng/ml and 0.0068 at 40ng/ml.

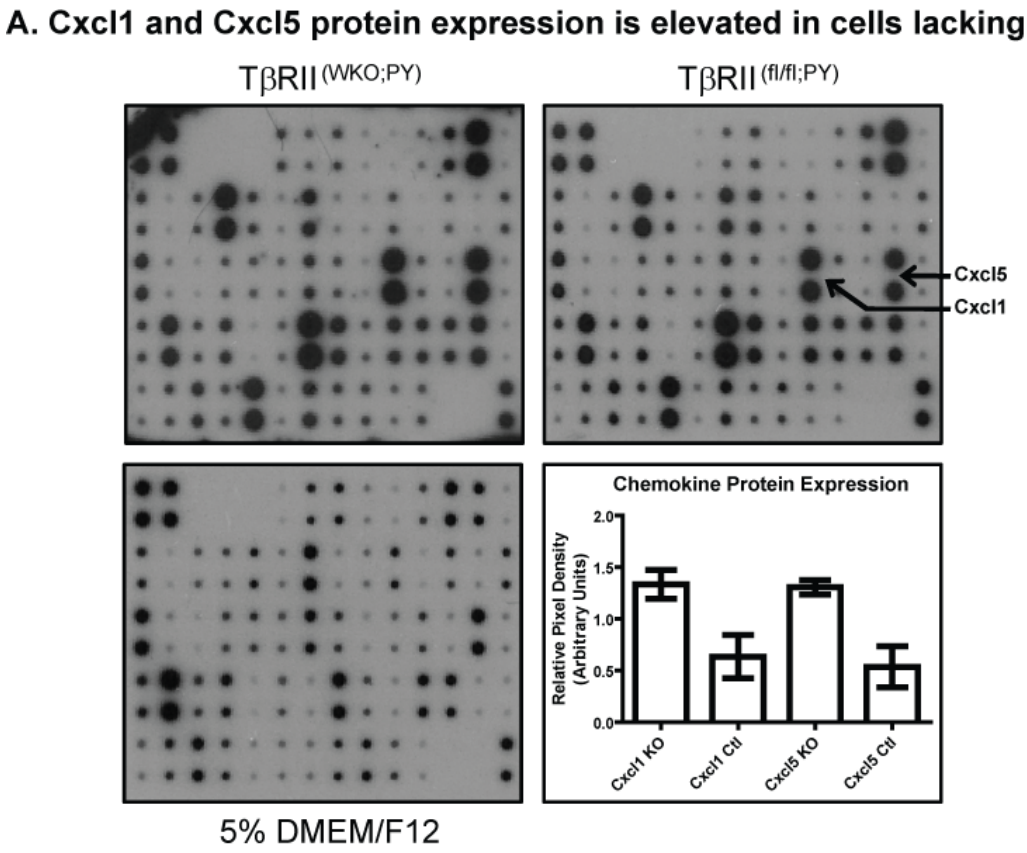


**Figure 23. TGF-β dependent chemokine protein secretion by mammary carcinoma cells and the effect of Cxcl1 stimulation on metastatic mammary carcinoma cell migration.**

**Table 4. Annotated legend for the Raybiotech Antibody Array.** The antibody array description was analyzed to determine alias designations for the included antibody antigens. Chemokine ligands were listed with their respective Ccl and Cxcl designations.

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pos | Pos | Neg | Neg | Blank | Axl | Blc | Cd30 L | Cd30 T | Cd40 | Cxcl10 | Ccl27b | Cxcl16 | Ccl11 |
| Pos | Pos | Neg | Neg | Blank | Axl | Blc | Cd30 L | Cd30 T | Cd40 | Cxcl10 | Ccl27b | Cxcl16 | Ccl11 |
| Ccl24 | Fasl | Cx3cl1 | Gcsf | Gm-csf | Ifng | Igfbp-3 | Igfbp-5 | Igfbp-6 | Il-1a | Il-1b | Il-2 | Il-3 | Il-3 Rb |
| Ccl24 | Fasl | Cx3cl1 | Gcsf | Gm-csf | Ifng | Igfbp-3 | Igfbp-5 | Igfbp-6 | Il-1a | Il-1b | Il-2 | Il-3 | Il-3 Rb |
| Il-4 | Il-5 | Il-6 | Il-9 | Il-10 | Il-12 p40/70 | Il-12 p70 | Il-13 | Il-17 | Cxcl1 | Leptin R | Leptin | Cxcl5 | L-Selectin |
| Il-4 | Il-5 | Il-6 | Il-9 | Il-10 | Il-12 p40/70 | Il-12 p70 | Il-13 | Il-17 | Cxcl1 | Leptin R | Leptin | Cxcl5 | L-Selectin |
| Lymphotactin | Ccl2 | Ccl12 | M-csf | Cxcl9 | Ccl3 | Ccl9 | Cxcl2 | Ccl19 | Ccl20 | Cxcl4 | P-Selectin | Ccl5 | Scf |
| Lymphotactin | Ccl2 | Ccl12 | M-csf | Cxcl9 | Ccl3 | Ccl9 | Cxcl2 | Ccl19 | Ccl20 | Cxcl4 | P-Selectin | Ccl5 | Scf |
| Ccl12 | Ccl17 | Ccl1 | Ccl25 | Timp-1 | TNFa | sTNFRI | sTNFRII | Tpo | Vcam-1 | Vegf | Blank | Blank | Pos |
| Ccl12 | Ccl17 | Ccl1 | Ccl25 | Timp-1 | TNFa | sTNFRI | sTNFRII | Tpo | Vcam-1 | Vegf | Blank | Blank | Pos |

Notably, *Cxcl1*, *Cxcl5*, *Bst2* and *Ctsa* were significantly upregulated in the TβRII$^{(WKO;PY)}$ cells when compared with the TGF-β treated controls. Based on our previous work with Cxcl5 (Bierie et al., 2008; Yang et al., 2008) and Chapter III, and the presence of *Cxcl1* in a lung metastasis gene expression signature identified in human breast cancer cells (Minn et al., 2005a), we selected these chemokines for further validation.

To examine the effect of our observed difference *Cxcl1* and *Cxcl5* mRNA expression with regard to protein secretion, we performed cytokine antibody array analyses with conditioned medium from the TβRII$^{(WKO;PY)}$ and control TβRII$^{(fl/fl;PY)}$ cell lines. Cxcl1 and Cxcl5 were the only secreted cytokines, out of sixty-two represented on the array, that were consistently differentially regulated when comparing the two models (Figure 23A and Table 4). The results paralleled those obtained in our real-time PCR validation of the microarray analyses. Previously we have shown that signaling through the Cxcr2 receptor, which is activated by both Cxcl1 and Cxcl5, was responsible for part of an enhanced inflammatory cell recruitment observed in association with the TβRII$^{(WKO;PY)}$ tumor microenvironment as compared to TβRII$^{(fl/fl;PY)}$ controls (Yang et al., 2008). However, the literature suggested that in addition to recruitment of inflammatory cell populations, Cxcr2 signaling may enhance the migration of carcinoma cells (Kawanishi et al., 2008; Li et al., 2004; Warner et al., 2008). To test this in mammary carcinoma cells we stimulated the highly metastatic murine 4T1 and human MDA-MB-231 cell lines with Cxcl1 at increasing doses in wound closure assays (Figure 23B). Our results indicated that carcinoma cell migration was enhanced in the presence of Cxcl1

stimulation, thereby providing another potential mechanism for enhanced metastasis when chemokine expression is elevated *in vivo*.

**TGF-β responsive genes were identified in TβRII$^{(fl/fl;PY)}$ mammary carcinoma cells**

Grouped analyses of the microarray results were able to identify genes that differed between the models, however a difference between the models did not necessarily equate to genes that were also TGF-β responsive. Out of the genes that we identified as differentially regulated by TGF-β (Table 2) we were able to validate 23 by real-time PCR (Table 5). As expected, many previously described TGF-β responsive genes were identified including *Serpine1*, *Smad6*, *PDGF-β*, *Lmcd1*, *Gadd45a* and *FosB*. In the list of differentially expressed genes that were validated by real-time PCR, the expression of *Tnfrsf1b* (tumor necrosis factor alpha receptor) was initially identified the TβRII$^{(WKO;PY)}$ *versus* TβRII$^{(fl/fl;PY)}$ profile. As previously mentioned this suggested that our conservative approach was effective in filtering genes for our TGF-β response signature. In addition to known TGF-β target genes, we were able to identify novel TGF-β responsive genes that may play a significant role in tumorigenesis. In particular, upregulation of *Map3k14* (NIK; NF-kB inducing kinase) by TGF-β was interesting since it has been shown that expression of this kinase alone was able to activate the NF-kB pathway. Importantly, the chemokines *Cxcl1*, *Cxcl5* and *Ccl20* were consistently suppressed by TGF-β. This data further substantiated and extended our previous preliminary analyses regarding TGF-β dependent regulation of host-tumor interactions associated with differential chemokine expression *in vitro* and *in vivo* (Bierie et al., 2008; Yang et al., 2008) and Chapter III.

**Table 5. Real-time PCR validation of TGF-β responsive genes in TβRII$^{(fl/fl;PY)}$ mammary carcinoma cells.** Each column represents 1/ΔCt values associated with an individually derived polyclonal control TβRII$^{(fl/fl;PY)}$ carcinoma cell line in the presence or absence of TGF-β at 10ng/ml for one hour. Genes that demonstrated the same direction of regulation were reported if all values were 1.5 fold or higher with a 2 fold or greater change in 2 out of 3 of the cell lines. Values were normalized to *Gusb*, *Hprt1*, *Hsp90ab1*, *Actb* and *PPIA*.

**Table 5. Real-time PCR validation of TGF-β responsive genes in TβRII$^{(fl/fl;PY)}$ mammary carcinoma cells.**

Upregulated by TGF-β (fold change)

| Gene Symbol | FL-1 | FL-2 | FL-3 |
|---|---|---|---|
| Camk2n1 | 7.3 | 4.7 | 3.3 |
| Cxcl12 | 5.7 | 2.6 | 1.8 |
| Cxcr4 | 3.9 | 3.4 | 1.7 |
| Edn2 | 13.5 | 3.8 | 2.5 |
| Egr2 | 3.1 | 2.7 | 2.0 |
| Egr3 | 4.1 | 2.4 | 1.8 |
| Fosb | 4.3 | 5.4 | 2.2 |
| Foxq1 | 7.8 | 2.6 | 1.7 |
| Gadd45a | 6.1 | 7.8 | 2.8 |
| Gja3 | 6.7 | 8.9 | 3.4 |
| Lmcd1 | 26.9 | 3.9 | 5.0 |
| Map3k14 | 15.2 | 3.0 | 2.5 |
| Pdgfb | 2.7 | 3.7 | 2.8 |
| Serpine1 | 12.0 | 16.8 | 8.3 |
| Smad6 | 12.9 | 4.3 | 4.1 |
| Tnfrsf1b | 3.4 | 3.1 | 1.7 |
| Wisp1 | 3.8 | 4.7 | 2.8 |
| Wnt9a | 3.2 | 3.1 | 2.5 |

Downregulated by TGF-β (fold change)

| Gene Symbol | FL-1 | FL-2 | FL-3 |
|---|---|---|---|
| Adamts1 | -14.3 | -7.5 | -3.1 |
| Ccl20 | -2.0 | -2.3 | -2.4 |
| Cxcl1 | -33.4 | -2.9 | -9.3 |
| Cxcl5 | -2.0 | -2.0 | -1.7 |
| Epgn | -16.0 | -3.7 | -2.0 |

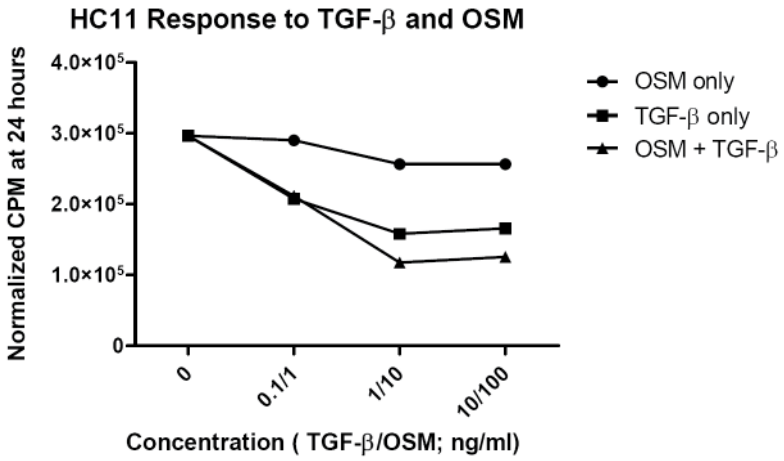


**Figure 24. TGF-β and OSM effect on HC11 cell growth.** Both TGF-β and OSM stimulation at varying concentrations resulted in decreased tritiated thymidine incorporation 24 hours after stimulation. TGF-β stimulation resulted in a significant decrease in tritiated thymidine uptake at 0.1, 1.0 and 10.0 ng/ml. OSM significantly decreased thymidine uptake at 10ng/ml and 100ng/ml. Results represent normalized mean counts per minute (CPM) +/- standard error of the mean. Significance was implied if the two-tailed unpaired t-test p-values were less than 0.05. The results for TGF-β and OSM appeared to be additive when both ligands were present in comparison with the values obtained from individual ligand stimulation.

**Figure 25. TGF-β attenuates basal and OSM induced expression Cxcl1, Cxcl5 and Ccl20 in mammary epithelium.** TGF-β (10ng/ml) and OSM (100ng/ml) stimulation was performed for one hour *in vitro*. Real-time PCR was performed using the HC11 (**A-D**, a) and NMuMG (**A-D**, b) cell lines. The median transformed 1/ΔCt values were reported as mean values +/- standard error of the mean (SEM). Differences when compared to the control were considered significant (**) if two-tailed, unpaired t-test p-values were less than 0.05. TGF-β significantly decreased *Cxcl1*, *Cxcl5* expression in HC11 and *Cxcl1*, *Cxcl5* and *Ccl20* in NMuMG cells (**A-C**, a and b). In the OSM responsive HC11 cell line, OSM significantly upregulated *Cxcl1*, *Cxcl5* and *Ccl20* expression (**A-C**, a). Importantly, TGF-β was able to significantly attenuate the effect of OSM with regard to *Cxcl1*, *Cxcl5* and *Ccl20* expression in the HC11 cell line. In the NMuMG cell line, which did not respond to OSM in growth or molecular response assays, did not demonstrate chemokine regulation by OSM (**A-C**, b). **D.** *Ccl5* expression was not altered by TGF-β or OSM treatment in the HC11 or NMuMG cell lines.

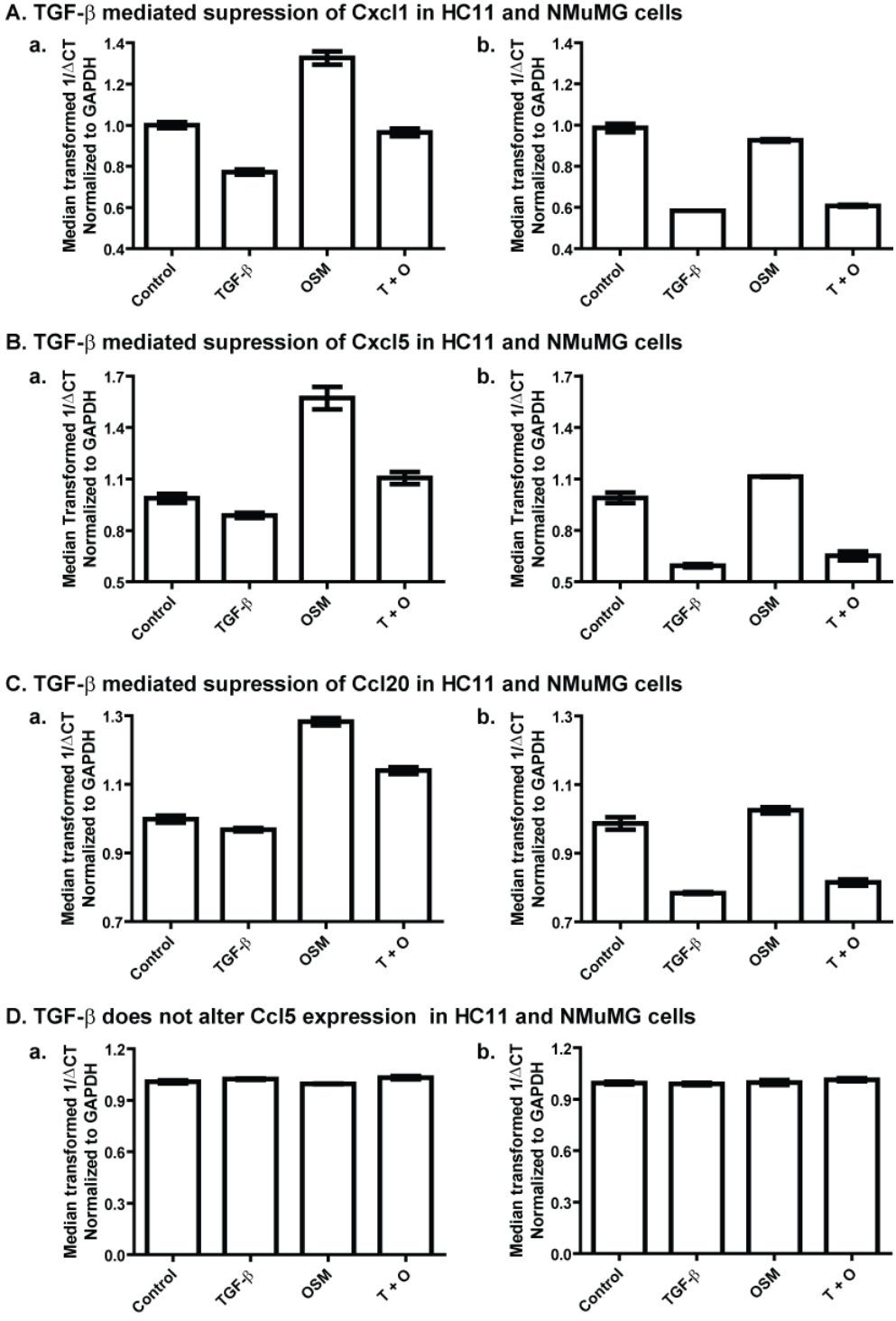


**Figure 25. TGF-β attenuates basal and OSM induced expression *Cxcl1*, *Cxcl5* and *Ccl20* in mammary epithelium.**

**TGF-β suppressed basal and Oncostatin-M (OSM) induced *Cxcl1*, *Cxcl5* and *Ccl20* expression in established mammary epithelial cell lines**

In our previous work, and in the current study, we have shown that TGF-β is able to suppress *Cxcl1*, *Cxcl5* and *Ccl20* expression in carcinoma cells that arise from mammary tissue in the MMTV-PyVmT mouse model. However, it was unclear if this regulation was specific for MMTV-PyVmT driven carcinoma cells or alternatively a general feature of TGF-β signaling in mammary epithelial cell populations. Therefore, we selected the non-transformed HC11 and NMuMG cell lines for analysis to address this issue. An important difference between the HC11 and NMuMG cell lines is that the HC11 cells do not express a functional p53 whereas the NMuMG cells have intact p53 signaling (Merlo et al., 1994; Rajan et al., 1996). Also, the NMuMG cells were able to activate p38 MAPK in response to TGF-β whereas the HC11 cells were not. Neither cell line demonstrated activation of the p44/42 pathway in response to TGF-β. To stimulate chemokine expression, we selected the OSM ligand due to its previously reported ability to upregulate *Cxcl1* and *Cxcl5* expression without a significant increase in *Ccl2* expression (Lafontant et al., 2006). Although this data was not produced in carcinoma cells it did demonstrate that in opposition to TNF-α, a ligand that is known to potently activate NF-Kb and thereby upregulate a large number of chemokines, OSM stimulation was more selective for regulation of the Cxcl1 and Cxcl5 chemokines that we had identified in our analyses. In addition, the presence of OSM in breast cancer is clinically relevant (Holzer et al., 2004; Jorcyk et al., 2006). OSM expression has been shown to be present in 66% of breast tumors (Crichton et al., 1996). Further, immunohistochemical analyses have been used to demonstrate that 35% of breast carcinomas and 88% of inflammatory breast cancers express this ligand (Holzer et al., 2004). The correlation

with inflammatory breast cancer is important since this disease is often highly aggressive and associated with poor patient prognosis (Lopez and Porter, 1996).

HC11 cells were responsive to OSM as determined by reduced tritiated thymidine uptake (Figure 24), and increased phosphorylation of signal transducer and activator of transcription 3 (Stat3) after stimulation. The HC11 cells were also responsive to TGF-β as determined by reduced tritiated thymidine uptake (Figure 24), and increased phosphorylation of Smad2 after stimulation. Importantly, TGF-β had no effect on OSM induced Stat3 phosphorylation and OSM had no effect on TGF-β dependent Smad2 phosphorylation. In HC11 cells, TGF-β and OSM treatment for one hour *in vitro* had a significant effect on *Cxcl1* expression (Figure 25A). In response to TGF-β for one hour *Cxcl1* expression was significantly decreased in HC11 cells. OSM treatment for one hour resulted in a significant upregulation of *Cxcl1* expression. Importantly, TGF-β treatment was able to significantly attenuate the effect of OSM with regard to regulation of *Cxcl1* expression. The results in HC11 cells were similar for *Cxcl5* (Figure 25B) and *Ccl20* (Figure 25C). The NMuMG monoclonal cell line that we have used for analysis responded well to TGF-β with regard to growth inhibition, induction of EMT and Smad2 phosphorylation. However, this clone did not respond to OSM stimulation with a growth response or Stat3 activation. Accordingly, TGF-β treatment for one hour significantly decreased the expression of *Cxcl1*, *Cxcl5* and *Ccl20* in the NMuMG cell line and OSM had no significant effect (Figure 25A-C). To determine if the observed regulation of *Cxcl1*, *Cxcl5* and *Ccl20* was selective, or alternatively a general chemokine effect, we analyzed the expression of *Ccl5* in response to TGF-β and OSM stimulation. *Ccl5*

mRNA was expressed by both cell lines, however no significant differences were observed in response to TGF-β, OSM or both ligands together (Figure 25D).

### The TβRII$^{(WKO;PY)}$ gene expression signature correlates with poor prognosis in human breast cancer

To determine the impact of our gene expression signatures in human breast cancer, we initially compared our TβRII$^{(WKO;PY)}$ and TGF-β treated gene expression signatures to the profiles obtained in the NKI295 dataset (Figure 26 and 27). Analysis of this comparison revealed a correlation with breast cancer subtype and reduced relapse-free survival when tumors had a gene profile similar to the TβRII$^{(WKO;PY)}$ signature. Therefore, to further extend and increase the power of our analysis, we subsequently compared our TβRII$^{(WKO;PY)}$ and TGF-β treated TβRII$^{(fl/fl;PY)}$ carcinoma cell signatures to microarray and survival data representing 1646 human breast cancer tissues (Figure 28A). The analyses revealed that the TβRII$^{(WKO;PY)}$ carcinoma signature correlated with Basal, HER2+ and Luminal B breast cancer subtypes more closely than with the Luminal A breast cancer subtype or Normal tissues (Figure 28A, top left). Importantly, the TβRII$^{(WKO;PY)}$ signature significantly correlated with a reduced ten year relapse-free survival (Figure 28A, top right). In contrast, the signature obtained from TGF-β treatment was correlated with the Luminal B tumor subtype, and more importantly, it did not correlate with a significant difference in relapse-free survival (Figure 28A, bottom left and right, respectively).

**Figure 26. Cluster analysis of the TβRII$^{(WKO;PY)}$ gene expression signature in the NKI295 dataset.** The results were the first to indicate that there may be an association between the TGF-β signaling deficient TβRII$^{(WKO;PY)}$ mammary carcinoma cell signature and subtype classification in human breast cancer.

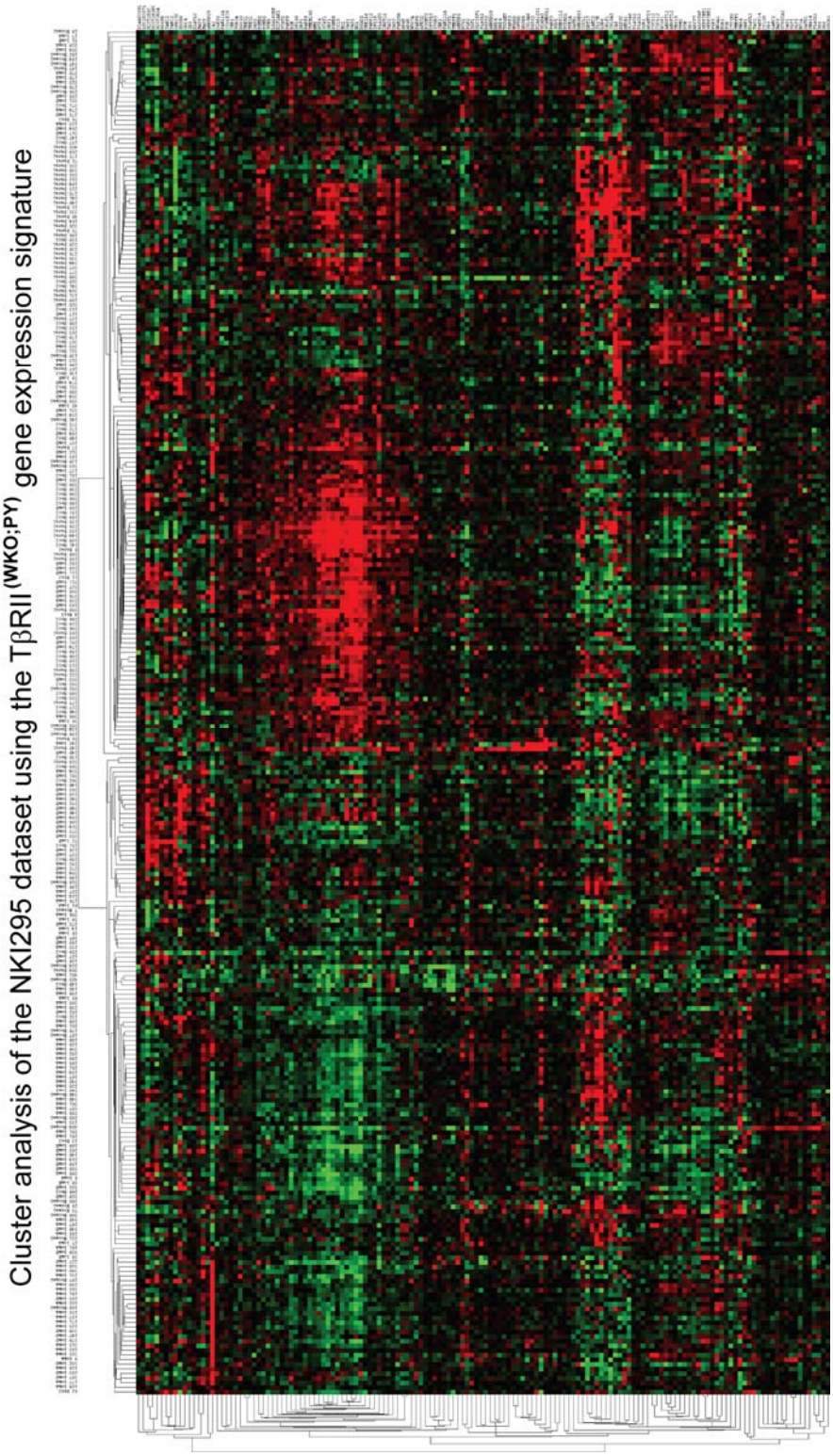


**Figure 26. Cluster analysis of the TβRII$^{(WKO;PY)}$ gene expression signature in the NKI295 dataset.**

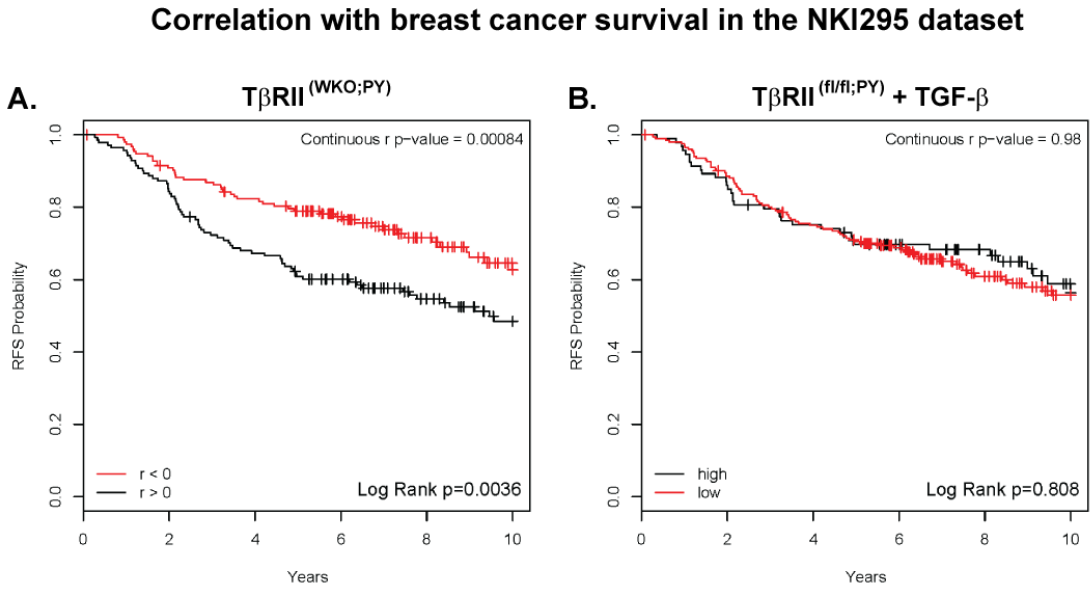


**Figure 27. Increased risk of poor prognosis when TGF-β signaling deficient TβRII$^{(WKO;PY)}$ mammary carcinoma cell gene expression signature correlated with samples within the NKI295 dataset.** **A.** The TβRII$^{(WKO;PY)}$ mammary carcinoma cell signature significantly correlated with reduced relapse-free survival (Continuous r p-value was 0.0008 and the Log Rank p-value was 0.0036). **B.** No significant correlation was noted between the TGF-β treatment gene expression signature and relapse-free survival.

**Figure 28. Loss of TGF-β signaling in mammary carcinoma cells resulted in a signature that correlated with tumor subtype and increased risk of relapse during human breast cancer progression. A.** TβRII$^{(WKO;PY)}$ and TβRII$^{(fl/fl;PY)}$ mammary carcinoma gene expression signatures were compared with profiles from 1646 human breast cancer tissues. The TβRII$^{(WKO;PY)}$ and TβRII$^{(fl/fl;PY)}$ + TGF-β treatment signatures were used to determine the correlation with previously described Basal, Her2, Luminal A or Luminal B tumor subtypes (left, top and bottom respectively). The TβRII$^{(WKO;PY)}$ signature was better at stratifying the tumors than the TβRII$^{(fl/fl;PY)}$ profile (ANOVA p-values were 8.4e-44 and 2.6e-08, respectively). The TβRII$^{(WKO;PY)}$ signature was more closely correlated with Basal, Her2 and Luminal B tumor subtypes than with the Luminal A subtype or Normal tissues. The TβRII$^{(WKO;PY)}$ and TβRII$^{(fl/fl;PY)}$ + TGF-β treatment signatures were also used to determine the correlation with relapse-free survival (right, top and bottom respectively). The TβRII$^{(WKO;PY)}$ signature significantly correlated with decreased relapse-free survival (Continuous r p-value was 0.018 and the Log Rank p-value was 0.0107). No significant difference in relapse-free survival was observed in correlation with the TGF-β treatment gene expression signature. **B.** In human lymph node positive (N+) breast cancer patients, the TβRII$^{(WKO;PY)}$ signature correlated with reduced relapse-free survival (left, Log Rank p-value was 0.0268) whereas the TGF-β treatment signature did not have a significant correlation (right).

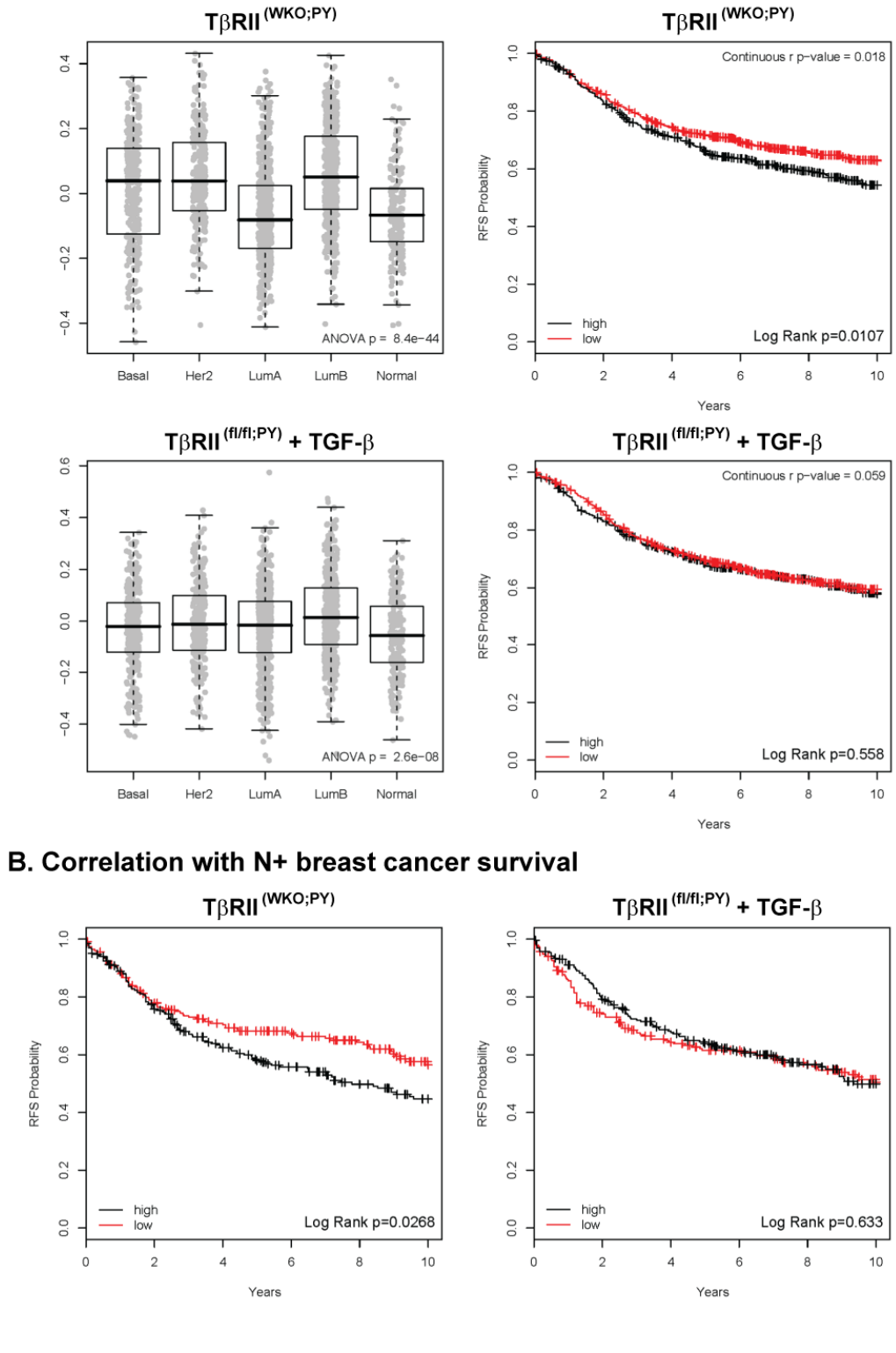


**Figure 28. TβRII$^{(WKO;PY)}$ and TGF-β signature correlation with human breast cancer subtype and relapse-free survival.**

**Figure 29. Increased risk of poor prognosis when human Luminal A, ER+ or hormone only treated breast cancer was associated with a TGF-β signaling deficient mammary carcinoma cell gene expression signature.** In human Luminal A, ER+ or hormone only treated breast cancer the $T\beta RII^{(WKO;PY)}$ signature correlated with a significant decrease in relapse-free survival (**A-C**, left; Log Rank p-values were 0.0188, 0.0005 and 0.0239 respectively). The TGF-β treatment signature did not correlate with a difference in relapse-free survival (**A-C**, right).

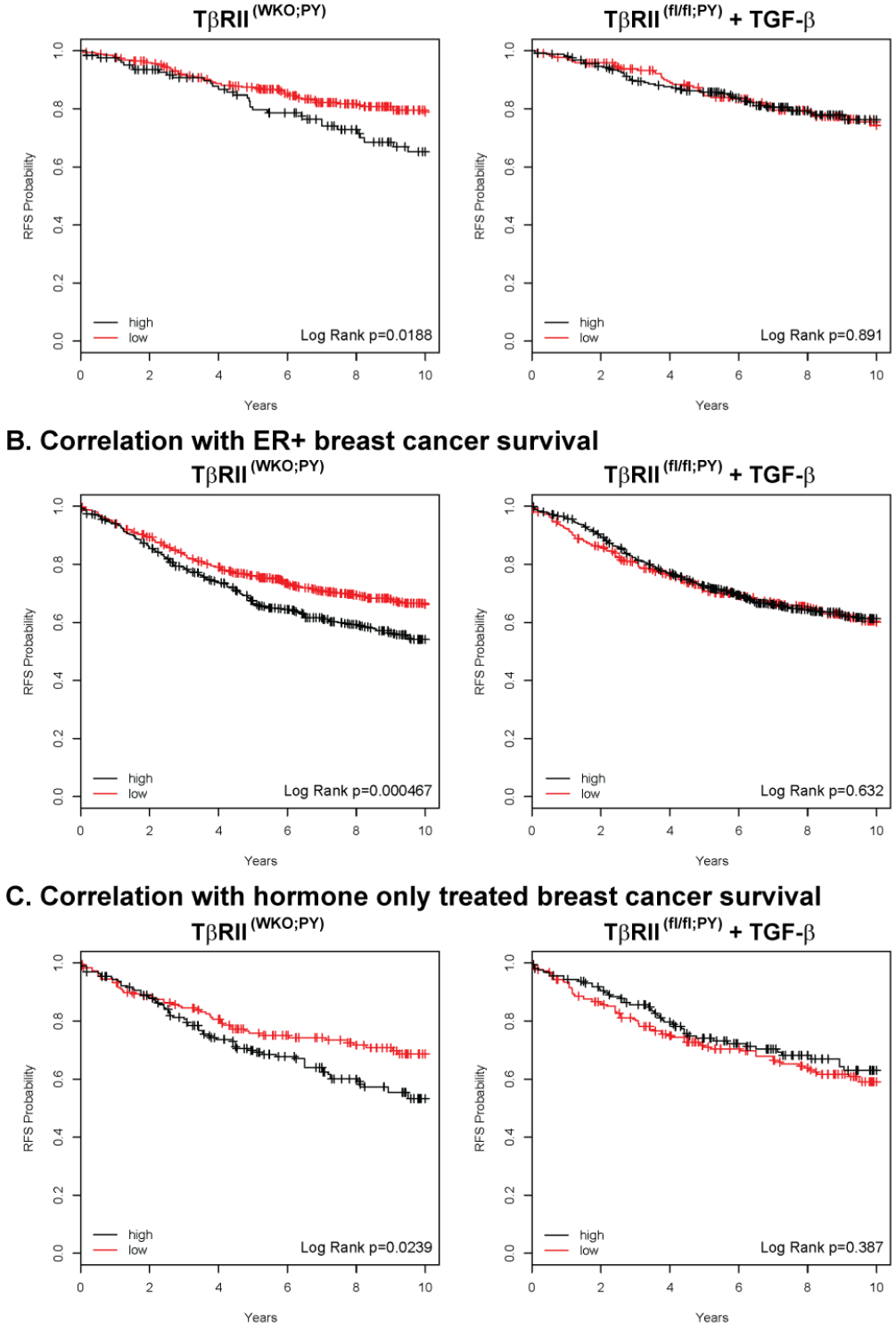


**Figure 29. TβRII$^{(WKO;PY)}$ and TGF-β signature correlation with human Luminal A, ER+ or hormone only treated breast cancer relapse-free survival.**

The TβRII$^{(WKO;PY)}$ signature significantly also correlated with a reduced ten year relapse-free survival in node positive breast cancer (Figure 28B, left) whereas the TGF-β treatment did not predict for a significant difference (Figure 28B, right). In node negative breast cancer, neither signature predicted for a difference in relapse-free survival. We further stratified the data based on primary tumor size at the time of tissue collection. In tumors that were less than two centimeters, there was not a significant difference for patient prognosis in correlation with the TβRII$^{(WKO;PY)}$ or TGF-β treatment signatures. In tumors that were larger than two centimeters the TβRII$^{(WKO;PY)}$ signature demonstrated a trend toward a correlation with reduced survival, however the p-value did not reach the 95% confidence interval that we had set for significance.

To further determine the impact of our TβRII$^{(WKO;PY)}$ gene expression signature we compared the results with individual categorical groups of gene profiles contained within the global human data set. In the human Luminal A group, our TβRII$^{(WKO;PY)}$ signature significantly correlated with a reduction in ten year relapse-free survival (Figure 29A, left). A significant correlation was not observed with regard to survival for either signature in association with human Luminal B and ER- tumors or those derived from patients that had not been treated with a systemic adjuvant. However, we observed a significant correlation between the TβRII$^{(WKO;PY)}$ signature and reduced ten year survival in human ER+ breast cancer (Figure 29B, left). In addition, we observed a significant correlation with reduced ten year relapse-free survival in the group of patients associated with hormone only treatment (Figure 29C, left). Alternatively the gene expression signature corresponding to TGF-β treatment did not predict for a difference in

relapse-free survival for the Luminal A, ER+ or hormone only treated patients (Figure 29A-C, right).

The TβRII$^{(WKO;PY)}$ and TGF-β treatment signatures were not correlated with a significant difference in survival for patients that had not been treated with a systemic adjuvant or Basal and Her2 breast cancer subtype patient populations. Together, our results suggest that TGF-β signaling may interact with the ER signaling network to regulate tumor recurrence during human breast cancer progression.

## Discussion

TGF-β is a well known regulator of tumor progression and metastasis, however it remains unclear how TGF-β actually regulates these processes. In early work, the focus of TGF-β signaling was on the tumor cell and a large amount of data supported an early tumor suppressive role for TGF-β in this context. However, it was also shown that TGF-β signaling within the carcinoma cell could promote tumor progression and metastasis. This dual role for TGF-β signaling has been referred to as the TGF-β paradox, and it is not known how TGF-β signaling switches from tumor suppressor to tumor promoter. However, our recent work demonstrating the recruitment of metastasis enhancing bone marrow derived cells to the tumor microenvironment could help explain this paradox. In addition to TGF-β signaling within the tumor cell, strong experimental evidence has also shown that an excess of secreted TGF-β could promote tumor progression. In our TβRII$^{(WKO;PY)}$ model of mammary carcinoma cell specific TGF-β signaling deficiency we observed a role for TGF-β as a suppressor of tumor initiation, progression and metastasis.

We observed differences in overall tumor burden, apoptosis, interaction with the adjacent fibrovascular stroma, angiogenesis, tumor cell heterogeneity and inflammation involving bone marrow derived cell populations (Bierie et al., 2008; Yang et al., 2008) and Chapter III. However, due to the complexity of the compound phenotype it was difficult to assess the molecular mechanisms of TGF-β signaling within the carcinoma cells that ultimately regulated the observed effects. Therefore, we established polyclonal carcinoma cell lines from our primary tumor tissues in order to more precisely determine the molecular differences present in the $T\beta RII^{(WKO;PY)}$ and $T\beta RII^{(fl/fl;PY)}$ models.

We subjected the carcinoma cells to microarray analyses and identified a relatively small number of genes that were differentially regulated when comparing the $T\beta RII^{(WKO;PY)}$ and $T\beta RII^{(fl/fl;PY)}$ models. Within this subset of genes, some of those we identified were previously described TGF-β responsive target genes. The identification of previously described TGF-β responsive genes in this analysis further substantiated the validity of our approach. Importantly, we were able to identify many genes that were differentially regulated between the two models that had not been previously reported. In addition, we were able to identify genes that were TGF-β responsive within one hour of stimulation. Together, these results provide an initial step toward identification of the essential molecular details related to mechanisms, including regulation of chemokine expression, that mediate TGF-β dependent control of tumor progression and metastasis *in vivo*.

Our previous work has shown that TGF-β within the carcinoma cell can regulate adjacent stromal-epithelial and host-tumor interactions within the tumor microenvironment (Bierie et al., 2008; Yang et al., 2008) and Chapter III. In addition,

our previously reported results and preliminary gene expression data suggested that the chemokines *Cxcl1*, *Cxcl5* and *Ccl20* were regulated by TGF-β in mammary carcinoma cells (Bierie et al., 2008; Yang et al., 2008) and Chapter III. Functionally, we were able to demonstrate that signaling through Cxcr2, a common receptor for Cxcl1 and Cxcl5, was responsible for a significant proportion of the enhanced immature bone marrow derived cell (myeloid immune suppressor cell; MISC) recruitment to the $T\beta RII^{(WKO;PY)}$ tumor microenvironment when compared to the $T\beta RII^{(fl/fl;PY)}$ controls (Yang et al., 2008). In our current study, we have now been able to demonstrate that these gene products are consistently suppressed by TGF-β in multiple independently derived $T\beta RII^{(fl/fl;PY)}$ mammary carcinoma cell lines. Upon TGF-β stimulation, suppression of *Cxcl1*, *Cxcl5* and *Ccl20* expression was a feature that significantly differentiated the $T\beta RII^{(WKO;PY)}$ and $T\beta RII^{(fl/fl;PY)}$ models. We have also been able to determine that the regulation of this chemokine subset by TGF-β is not specific for the MMTV-PyVmT transformed cell populations. In the non-transformed HC11 and NMuMG mammary epithelial cell lines, TGF-β significantly suppressed *Cxcl1*, *Cxcl5* and *Ccl20* expression. In the absence of TGF-β treatment, OSM stimulation increased the expression of these chemokines. Since OSM presence is clinically relevant in breast cancer (Crichton et al., 1996; Holzer et al., 2004; Jorcyk et al., 2006), the link we have identified with TGF-β signaling is likely important. Specifically, it has been shown that a majority of inflammatory breast cancers express this ligand (Holzer et al., 2004), and our current results suggest that TGF-β and OSM dependent chemokine production may have a functional role in the regulation of this type of breast cancer that is often aggressive and associated with poor patient prognosis (Lopez and Porter, 1996). Further, *Cxcl1* expression was recently correlated

with a metastasis gene expression signature in human breast cancer (Minn et al., 2005b). Together, the data suggest that TGF-β can suppress basal and OSM induced *Cxcl1*, *Cxcl5* and *Ccl20* expression in non-transformed mammary epithelium and in carcinoma cells thereby regulating inflammation that is known to promote tumor progression and metastasis.

The power of molecular profiling has been highlighted in recent years, since it has been shown to effectively predict for mammary tumor subtype and relapse-free survival over time (Carroll et al., 2006; Ivshina et al., 2006; Loi et al., 2007; Loi et al., 2008; Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003; van 't Veer et al., 2002; van de Vijver et al., 2002; Wang et al., 2005b). According to the literature, many of the genes that we identified in this study may have a significant role in the regulation of tumor progression and metastasis (Table 1 and 2). However, the literature at the level of gene by gene analysis often lacks the ability to predict interactions between signaling networks that ultimately regulate the process of tumor progression and metastasis *in vivo*. Further, it is not feasible to concurrently upregulate roughly two-hundred genes and downregulate seventy others in a single cell to experimentally model the compound networking interactions *in vivo*. Therefore, we used the gene expression signatures obtained in our current study to compare with the individual profiles obtained in five previously described human breast cancer data sets representing 1646 breast cancer patients (Carroll et al., 2006; Ivshina et al., 2006; Loi et al., 2007; Loi et al., 2008; van 't Veer et al., 2002; van de Vijver et al., 2002; Wang et al., 2005b). The patient data included tumor subtype classification, size, lymph node status, ER status, treatment regimen and survival over a ten year period. Importantly, we were able to determine that the $T\beta RII^{(WKO;PY)}$ signature

predicted for reduced relapse-free survival in lymph node positive, ER+ or tamoxifen only treated patients. There was also a trend toward reduced relapse-free survival in patients that presented with primary tumors larger than two centimeters and exhibited a gene expression signature that correlated with the $T\beta RII^{(WKO;PY)}$ profile. Notably, the TGF-β treatment gene expression signature did not predict for a difference in relapse-free survival in any of the correlative analyses that we conducted.

In summary, we have now been able to identify significant molecular differences in mammary carcinoma cells lacking the ability to respond to TGF-β when compared to mammary carcinoma cells that are capable of a TGF-β response. The molecular signatures suggested that intrinsic, stromal-epithelial and host-tumor interactions together mediate tumor progression and metastasis as a result of the tumor cell response to TGF-β stimulation. Further at the time of diagnosis in human breast cancer, if a molecular signature that resembles the carcinoma cell specific TGF-β signaling deficiency is detected in Luminal A subtype, ER+ and lymph node positive tumors, the corresponding patients may benefit from an aggressive post-operative treatment regimen to increase their chance for relapse-free survival.

# CHAPTER V

# DISCUSSION AND FUTURE DIRECTIONS

My work presented herein has primarily focused on the role for normal epithelial and carcinoma cell response to TGF-β signaling *in vivo* and *in vitro*. The results have led to the identification of intrinsic, stromal-epithelial and host-tumor interactions that significantly regulate normal mammary development and tumorigenesis. Notably, the results demonstrate that the regulation of tumorigenesis by carcinoma cell specific responses to TGF-β signaling can promote carcinoma cell apoptosis, interaction with the adjacent fibrovascular stroma, decreased tumor cell heterogeneity, suppression of inflammatory gene expression and suppression of metastasis enhancing bone marrow derived cell recruitment. Further, upon loss of signaling through this pathway in mammary carcinoma cells, a global gene expression profile was induced that correlated with increased risk of relapse for patients that had Luminal A, ER+ or lymph node positive tumors at the time of tissue collection. Together, these studies have made a significant step forward with regard to our understanding of the complex role for TGF-β signaling during normal mammary development and subsequent tumorigenesis. The following text will review the previous literature to put our findings in context and discuss the impact of our results with regard to human disease.

## The TGF-β superfamily

The TGF-β superfamily now contains around 40 secreted ligands that potently regulate cell growth and differentiation through heteromeric signaling complexes composed of TGF-β type I and type II receptors (TGFβ-RI and TGFβ-RII respectively). The TGFβ-RIs, activin-like receptors 1-7 (Alk-1, Alk-2, Alk-3, Alk-4, Alk-5, Alk-6 and Alk-7), pair with specific TGFβ-RIIs (TβRII, BmpRII, ActRII, ActRIIB and AmhRII) to transduce signals from TGF-β superfamily ligands (Shi and Massague, 2003). TGF-β1, TGF-β2 and TGF-β3 signal through a TβRII in association with Alk-1, Alk-2 or Alk-5. In most cell types, the TβRII/Alk-5 complex transduces the signal from TGF-β1, TGF-β2 and TGF-β3 whereas TβRII also associates with Alk-1 in endothelial cells and Alk-2 in cell types related to cardiovascular development (Derynck and Zhang, 2003; Desgrosellier et al., 2005; Lai et al., 2000; Lebrin et al., 2005; Miettinen et al., 1994; Olivey et al., 2006).

Important differences in the alternate heteromeric TβRII signaling complexes initiated by TGF-β involves activation of alternate Smad family members. In general, the Smad family of proteins influences cell behavior through transcriptional regulation of gene expression. Alk-1 and Alk-2 activate receptor associated Smad (R-Smad) proteins Smad1, Smad5 and Smad8, while Alk-5 activates Smad2 and Smad3 (Derynck and Zhang, 2003; Desgrosellier et al., 2005; Lai et al., 2000; Lebrin et al., 2005; Miettinen et al., 1994; Olivey et al., 2006). The Alk-5 kinase domain specifically mediates downstream signal transduction in response to TGF-β stimulation, however it is structurally similar to Alk-4 and Alk-7 kinase domains. The structural similarity between the Alk-4, Alk-5 and Alk-7 kinase domains contributes to the observed inhibition of Alk-

4 and Alk-7 using small molecule inhibitors designed to attenuate Alk-5 kinase activity (Peng et al., 2005). Although similar to Alk-5 structurally, the Alk4 and Alk-7 type I receptors preferentially bind the activin type II-B receptor, ActR-IIB, and display extracellular domains that are specific for alternate TGF-β family ligands. The Alk-4 receptor binds ActR-IIB in the presence of inhibin ligands whereas the Alk-7 binds the ActR-IIB receptor in the presence of Nodal, Gdf-1 or Vg1. In addition, the Alk-7/Act-RIIB receptor pair has been shown to bind the accessory receptor Cripto. Importantly, both Alk-4 and Alk-7, like Alk-5 activate downstream Smad 2 and Smad3 signaling (Shi and Massague, 2003).

## TGF-β dependent regulation of mammary development

To understand how TGF-β regulates tumorigenesis, it is essential to also understand how signaling through this pathway regulates normal mammary development. During the process of normal mammary development, the three TGF-β ligands appear to be functionally redundant and when present they are able to significantly inhibit epithelial cell growth. The developmental regulation derived from TGF-β signaling *in vivo*, was initially demonstrated through administration of slow release TGF-β1, TGF-β2 or TGF-β3 implants within the mammary fat pad (Robinson et al., 1991; Silberstein and Daniel, 1987). The regulation of epithelial cell growth by TGF-β in these experiments resulted in the reduction of lateral ductal branching, decreased proliferation in the mammary stem cell associated endcaps and increased involution in the ductal endbuds. One of the earliest studies *in vivo*, involving transgenic expression of TGF-β in mice, featured the production of a TGF-β ligand targeted to the mammary gland under control of the

MMTV promoter/enhancer (MMTV-TGF-β1) (Pierce et al., 1993). In this study, it was shown that expression of TGF-β1 by the mammary epithelium resulted in hypoplastic growth of mammary ducts. In an effort to understand the role for TGF-β at later points during functional differentiation in the mammary gland, the TGF-β1 ligand was expressed under control of the *whey acidic protein* (*WAP)* gene promoter, and the result was a decrease in the formation of lobular alveolar structures, milk protein expression and premature stem cell senescence (Boulanger et al., 2005; Jhappan et al., 1993; Kordon et al., 1995). The data again suggested that TGF-β had a role in suppressing epithelial growth and also suggested that it had a role in regulation of milk protein gene expression. In similar studies, the TGF-β3 ligand was shown to have an impact on mammary involution when expressed from mammary epithelium under control of the *beta-lactoglobulin (BLG)* gene promoter (Nguyen and Pollard, 2000). However, we have now been able to identify an additional feature of TGF-β signaling during mammary involution that suggests that signaling through this pathway is essential for suppression of terminal differentiation during late stages of this process in order to completely remodel the mammary gland. The previously described apoptotic role for TGF-β signaling was shown to be prevalent during the first three days of involution. However, commitment to cell death is not thought to be as prevalent at this reversible stage of involution as it is during the second irreversible stage of mammary involution. Importantly, our data demonstrates that in the complete absence of epithelial cell response to TGF-β signaling, there is not a significant shift in the timing of commitment to the second irreversible stage of mammary involution. Rather, it appears that after initiation of the second stage of involution, TGF-β signaling is essential for suppression of spontaneous terminal

differentiation. Together, our results suggest that in addition to cytostatic and apoptotic control of mammary epithelial cell behavior, TGF-β signaling can regulate the mammary epithelial cell response to endocrine signaling during late stages of mammary involution *in vivo*.

Previous work in the field have been able to demonstrate clear links between the TGF-β pathway and endocrine signaling associated with mammary epithelium. One of the first studies to suggest a role for TGF-β signaling demonstrated that when expressed under control of the *Wap* promoter, TGF-β1 resulted in normal ductal development with impaired lobular alveolar differentiation and subsequent deficiencies in the ability to lactate (Jhappan et al., 1993). Later, it was suggested that TGF-β restrains the response of differentiated mammary epithelium to systemic hormones in correlation with estrogen receptor-alpha (ER-α) expression (Ewan et al., 2005). In this study, mice that were heterozygous for *Tgfb1* were used to determine the rate of estrogen receptor-alpha positive mammary epithelial cell proliferation during estrus. Loss of TGF-β signaling resulted in an increased number of ERα+ cell populations *in vivo*. The loss of function experiments were complemented by gain of function experiments through overexpression of TGF-β1 under control of the MMTV promoter/enhancer. Increased TGF-β expression resulted in a decreased number of ERα+ cells within the mammary tissues. The results, in both systems demonstrated that activation of the TGF-β pathway negatively regulated estrogen receptor positive mammary epithelial cell proliferation *in vivo* (Ewan et al., 2005).

Previously it has been shown that TGF-β and Prolactin (Prl) exert opposing effects on mammary epithelial cell survival (Bailey et al., 2004), however this

observation did not exclude the effect of Prl on endocrine hormone production that may have had a significant impact on mammary development and function *in vivo*. The difficulty of interpreting the prolactin impact in correlation with TGF-β signaling is complicated by previous work demonstrating that prolactin can act as a systemic stimulant for production of endocrine effectors *in vivo*. Importantly, this includes the production of estrogen that is known to significantly interact with TGF-β signaling to regulate mammary development. The importance of systemic regulation of endocrine signaling was demonstrated in early studies related to this process *in vivo*. Systemic administration of prolactin was sufficient to reduce mouse mammary epithelial cell apoptosis during involution (Sheffield and Kotolski, 1992). However, local delivery of prolactin using slow release pellet implants was shown to exert no effect on mammary involution (Feng et al., 1995). The results associated with these studies supported a hypothesis postulated nearly thirty years ago wherein systemic prolactin was thought to enhance endocrine factors that subsequently regulate mammary involution whereas local prolactin stimulation alone would not be sufficient for regulation of this process *in vivo* (Ossowski et al., 1979).

The regulation of mammary development by TGF-β and prolactin signaling is further complicated by data demonstrating that the observed effect of estrogen stimulation involves a mammary stromal response through paracrine signaling that significantly regulates the adjacent epithelium (Cunha et al., 1997; Mueller et al., 2002). The significant effect of mammary stroma on adjacent epithelial cell fate and differentiation has been known for many years. In early studies, embryonic mesenchyme was shown to regulate the fate of mammary epithelial cell progenitors (Topper and

Freeman, 1980). In this early work, mammary anlagen when recombined with mammary mesenchyme produced outgrowths that resembled normal mammary tissue with ductal structures and terminal end buds. However, if the embryonic mammary anlagen was recombined with salivary mesenchyme the progenitors formed adenomere-like structures resembling those typically associated with salivary tissue (Topper and Freeman, 1980). The paracrine stromal-epithelial relationship involving ERα was clearly shown in experiments that demonstrated mammary epithelial ducts could be formed in the absence of epithelial cell specific expression of ERα (Mueller et al., 2002). When stimulated with estrogen and progesterone, the stromal response was sufficient to promote the full effect normally observed with regard to adjacent epithelial cell proliferation *in vivo*. However, in the absence of stromal ERα no mammary epithelial growth was observed unless the mice were treated with high levels of estrogen and progesterone. Importantly, only a rudimentary ductal system was observed with stromal loss of ERα, whereas full lobular alveolar proliferation was observed when ERα was present in the stroma (Mueller et al., 2002). The stromal contribution to ERα signaling was similar to progesterone receptor results attained through tissue recombination experiments. Transplanting PR null mammary epithelium into a wild type host demonstrated that a significant response to progesterone is dependent upon epithelial cell specific PR expression during mammary development (Humphreys et al., 1997). Interestingly, using the reciprocal transplant approach, this study also revealed a requirement for stromal PR expression to promote proper ductal extension within developing mammary tissue. Together, these results and supporting literature suggest that prolactin mediates the production of endocrine factors

that subsequently interact with the TGF-β pathway to regulate terminal differentiation of mammary epithelium *in vivo*.

Our current results highlight the role for TGF-β signaling in the suppression of terminal differentiation in mammary epithelium during post-lactation involution. Over the years we and others have made observations related to the apparent delay of mammary involution in our mouse models that have mammary epithelial cell specific attenuation of TGF-β signaling. In the past, our interpretation has been that TGF-β has an effect on the involution process through inhibition of apoptosis in mammary epithelium. However, we now realize that the observed phenotypes likely represent TGF-β dependent regulation of at least two distinct processes that correspond with mammary involution: regulation of apoptosis during the first phase of involution and perhaps more importantly suppression of terminal differentiation late during the second phase of involution.

Despite well documented regulation of apoptosis during the first three days of mammary involution, our results demonstrate that many of the processes that should occur during transition from the first-to-second phase of involution are not significantly altered when TGF-β signaling is completely abrogated in mammary epithelium (Chapter II). This juncture normally denotes the timepoint when mammary involution changes from a reversible to irreversible process *in vivo*. During the second stage of mammary involution, activation of caspase and metalloproteinase proteins are known to correlate with significant mammary epithelial cell death. Mammary epithelial cell death during the second phase of involution normally proceeds until the gland is completely remodeled, thereby resembling a virgin-like state. However, in our tissues with ablated TGF-β

signaling in mammary epithelium, terminal differentiation is silenced at the transition point then subsequently re-initiated late during this process. These results and others presented in our current work (Chapter II), demonstrate that suppression of terminal differentiation by TGF-β is essential for remodeling of mammary tissue during post-lactation involution *in vivo*.

Importantly, the previous literature does not accurately represent the dual role for TGF-β during mammary involution, and the work presented herein makes a significant step forward with regard to addressing this concept *in vivo* (Chapter II). Specifically, we have previously shown that mammary epithelial cell specific loss of the type II TGF-β receptor (TβRII) results in selective ablation of TβRII deficient cell populations *in vivo*. To subvert complications in analysis that arise from the gradual loss of TβRII deficient cell populations we have now targeted TβRII ablation within mammary epithelium using the WAP-Cre transgene [TβRII$^{(WKO)}$Rosa26R]. Interestingly, our results demonstrated that ablation of TGF-β signaling did not have a significant impact on several key processes during the transition from first to second phase of mammary involution. Importantly, at day three of mammary involution the Na–Pi type IIb co-transporter (Npt2b), a selective marker for active lactation in luminal lobular alveolar epithelium, was completely silenced in the WAP-Cre control and TβRII$^{(WKO)}$Rosa26R tissues. However, by day seven of involution the TβRII$^{(WKO)}$Rosa26R tissues had distended lobular alveoli with robust Npt2b expression at the apical luminal surface. The Npt2b expression and corresponding increase in *WAP* mRNA expression, suggested that the expansion was due to re-initiation of an active lactation program rather than the result of residual milk protein and lipid accumulation. In summary, our current results suggest

that a critical function for TGF-β signaling in mammary epithelium during the second stage of involution and remodeling is suppression of spontaneous terminal differentiation *in vivo*.

## Alterations of the TGF-β pathway in human cancer

The TGF-β ligands (TGF-β1, TGF-β2 and TGF-β3), receptors and downstream signaling components have been the subject of a large number of studies involving cancer over the past two decades. It is now generally accepted that the transforming growth factor beta ligands TGF-β1, TGF-β2 and TGF-β3 are potent regulators of cell growth, differentiation and migration (Derynck and Zhang, 2003; Siegel and Massague, 2003). A large number of studies have identified mutations in components associated with the TGF-β pathway that correlate with cancer occurrence and prognosis in a diverse array of human tissues (Levy and Hill, 2006). In human, overexpression of TGF-β1 has been associated with breast, colon, esophageal, gastric, hepatocellular, lung and pancreatic cancer (Bierie and Moses, 2006a; Levy and Hill, 2006). Importantly, the overexpression of TGF-β in human cancer has been correlated with tumor progression, metastasis, angiogenesis and a poor prognosis (Bierie and Moses, 2006a; Levy and Hill, 2006). However, TGF-β mediated signaling has a dual role in the regulation of cancer; initially as a tumor suppressor and then as the tumor develops, as a positive mediator of tumor progression. Retrospective studies have revealed that in various tumor types, *TGFBR1*, *TGFBR2*, *SMAD2* and *SMAD4* are commonly inactivated through mutation or allelic loss of heterozygosity (Bierie and Moses, 2006a; Grady, 2004; Levy and Hill, 2006;

Markowitz et al., 1995; Pasche, 2001). In addition to these analyses, common *TGFBR2* mutations in the 10bp adenine repeat (polyA$_{10}$) region have been introduced experimentally through controlled induction of microsatellite instability (MSI), providing a causal relationship between MSI and the reported observations of *TGFBR2* polyA$_{10}$ mutations in human cancer (Bacon et al., 2001). Furthermore, transcriptional repression and DNA methylation of *TGFBR1* and *TGFBR2* also occur in human cancer (Kang et al., 1999; Kim et al., 2000). Transcriptional repression of *TGFBR2* is particularly important, since it appears that this mode of regulation may be responsible for most of the tumor associated TGF-β resistance observed *in vivo* (Kim et al., 2000). Retrospective studies, though informative, do not provide a mechanistic insight into the role for TGF-β signaling in cancer. Most of the recent valuable insights into the function of the TGF-β pathway in tumorigenesis have come from mouse models.

## Mouse models permit the study of TGF-β signaling *in vivo*

Many elegant systems have been used to demonstrate that TGF-β can directly contribute to the regulation of tumor cell autonomous signaling, immune evasion, angiogenesis, fibroblast-to-myofibroblast transdifferentiation and stromal-epithelial crosstalk (Akhurst and Derynck, 2001; Bierie and Moses, 2006a; Derynck et al., 2001; Lebrin et al., 2005; Siegel and Massague, 2003). Importantly, the use of mouse models has enabled the dissection of the function for TGF-β in tumor development and progression when TGF-β is expressed by the tumor cells themselves, or when it is expressed by the cellular components of the surrounding tumor stroma. Moreover, these models have been used to differentiate between the effect of TGF-β on epithelial cells,

the established tumor cells or on the surrounding stromal cells. In this respect, experimental mouse models related to breast and skin development have been particularly useful. However, homozygous *Tgfβ1*, *Tgfβ2*, *Tgfβ3*, *Tgfbr1* or *Tgfbr2* deletions in mice are lethal (Pangas and Matzuk, 2004), and for this reason manipulation of the TGF-β pathway has been largely achieved through transgene expression or conditional null mutations *in vivo*. The transgenes used modify the TGF-β pathway in various ways including overexpression of the ligand or production of a dominant active or negative receptor — usually a dominant active TβRI or a dominant negative TβRII — under the control of a tissue specific promoter.

The function of TGF-β in breast development and cancer has been studied using various mouse models. Many of the models use a mouse mammary tumor virus (MMTV) promoter/enhancer to drive expression of transgenes in the mammary gland epithelium throughout development, pregnancy, lactation and involution. Other models use the *whey acidic protein* (*WAP*) promoter to drive expression of transgene in the mammary lobular-alveolar epithelium beginning around day 13 of pregnancy through to the second day of post-lactation involution. Many of these mouse models have been cross bred with transgenic mice that have a predisposition towards the development of breast tumors, such as the MMTV-c-*Neu* also known as *ErbB2* mice, MMTV-TGFα mice and MMTV-polyoma virus middle T antigen (PyVmT) mice (Guy et al., 1992a; Guy et al., 1992b; Matsui et al., 1990). In addition to transgene expression, chemically induced carcinogenesis is used, though with less frequency, to induce mammary tumors in mice. Studies of skin carcinogenesis in mice often use defined chemicals to induce skin tumors and this is a reliable method with which to study the effect of TGF-β signaling *in vivo*.

**Functional effects of mammary epithelial cell derived TGF-β production on tumor initiation and progression**

The mammary gland can develop from a single stem cell producing ductal, lobular-alveolar and myoepithelial cell types that proceed through several distinct stages including virgin, pregnancy and lactation followed by a post-lactation involution that returns the gland to a virgin-like state (Hennighausen and Robinson, 2005; Shackleton et al., 2006). TGF-β signaling can potently regulate the mammary stem cell and differentiated epithelial cell populations *in vivo* (Boulanger and Smith, 2001; Boulanger et al., 2005; Jhappan et al., 1993; Joseph et al., 1999; Kordon et al., 1995; Nguyen and Pollard, 2000; Pierce et al., 1993). Expression of a constitutively active TGF-β1 protein (containing two site specific mutations that replace cysteines with serine residues at positions 223 and 225 to prevent binding of the latency associated peptide (LAP) that would otherwise inactivate the mature TGF-β1 protein product), when placed under the control of the MMTV promoter/enhancer, resulted in the development of mammary ductal hypoplasia (Lebrin et al., 2005). Although ductal development was impaired in these mice, lobular-alveolar structures were able to develop sufficiently to sustain the survival of full litters during lactation. Mammary epithelium-specific expression of TGF-β1 under control of the *WAP* promoter (WAP-TGF-β1) also resulted in inhibition of lobular-alveolar structures and reduced milk protein expression (Jhappan et al., 1993). Premature aging of the mammary epithelial cells was evident in this mouse model and this has been suggested to be the result of TGF-β-induced stem cell senescence (Boulanger et al., 2005; Kordon et al., 1995). These initial studies indicated that overexpression of TGF-β in the mammary epithelium is growth inhibitory and suggested that it might have a tumor suppressive effect in cancer.

The first study to clearly address the function of TGF-β in mammary carcinoma development *in vivo* used MMTV-TGF-β1 transgenic mice either crossed with MMTV-TGF-α transgenic mice, or MMTV-TGF-β1 mice treated with 7,12-dimethylbenz[a]anthracene (DMBA) to induce mammary tumors (Pierce et al., 1995). The time taken for the tumors to develop was increased in the bigenic mouse model compared with the mice expressing TGF-α alone. In addition, the MMTV-TGF-β1 transgenic mice were resistant to DMBA-induced mammary tumorigenesis, and the induction of breast tumors induced by injecting an active form of MMTV, was inhibited in WAP-TGF-β1 transgenic mice (Boulanger and Smith, 2001). Interestingly, when the MMTV-TGF-β1 transgenics were crossed with MMTV-c-*Neu* transgenics, there was no difference in the time taken for the tumors to develop in the bigenic *versus* the single transgenic animals (Muraoka et al., 2003). However, tumors from the bigenic mice demonstrated higher levels of vimentin expression compared with the MMTV-c-*Neu* controls. Increased vimentin expression is associated with increased cellular mobility and tumors from the bigenic animals were more invasive and metastatic than in the MMTV-c-*Neu* controls. Finally, expression of a tetracycline-inducible (tet-op$_7$) MMTV-TGF-β transgene for 2 weeks in MMTV-PyVmT;tet-op$_7$ MMTV-TGF-β mice with established breast tumors produced a ten fold increase in the number of lung metastases, while no effect was observed in primary tumor size or rate of cell proliferation with the induction of TGF-β expression (Muraoka-Cook et al., 2004). Together the results from these studies involving the expression of TGF-β by epithelial cells indicate that TGF β has a role in early tumor suppression but later it can contribute to tumor progression.

**Mammary epithelial cell response to TGF-β signaling can regulate tumor initiation and progression**

The studies above increased our understanding of how epithelial cell derived TGF-β production could influence mammary development and tumorigenesis, but they did not specifically address the response of the epithelial cells themselves to TGF-β expression (the cell autonomous response) *in vivo*. This has been addressed through the expression of dominant active and dominant negative TGF-β receptors *in vivo*. Expression of the dominant negative *Tgfbr2* transgene (MMTV-DNIIR) in mammary epithelium resulted in alveolar hyperplasia and differentiation in virgin mice, precocious differentiation during early pregnancy, and unlike the TGF-β transgenic mice, mammary gland development during late pregnancy and lactation were impaired, and involution was delayed (Gorska et al., 2003). MMTV-DNIIR mice develop spontaneous tumors at approximately 27.5 months of age, and when crossed with MMTV-TGF-α mice, the time taken for the tumors to develop and incidence of invasion were decreased. The invasive tumors, when they did occur, showed that expression of the MMTV-DNIIR transgene was repressed, indicating that re-establishment of the TGF-β signaling pathway was selected for in these invading tumor cells. Precocious lobular-alveolar differentiation and aberrant proliferation was also observed in mice that express antisense *Tgfbr2* under control of the MMTV promoter/enhancer, further indicating that the inhibition of TGF-β signaling in mammary epithelium could result in enhanced growth (Lenferink et al., 2003). In another study using a different dominant negative TβRII receptor construct (MMTV-TβRII$^{\Delta Cyt}$), transgenic mice were crossed with MMTV-c-*Neu*$^{YB}$ and MMTV-c-*Neu*$^{YD}$ transgenic mice to induce mammary tumors (Siegel and Massague, 2003). The modified *Neu* transgenes activate only a restricted number of downstream signaling

molecules that are dependent on the binding of the adaptors molecules GRB2 (YB) or Shc (YD). Crossing TβRII$^{\Delta Cyt}$ mice with either MMTV-c-*Neu*$^{YB}$ or MMTV-c-*Neu*$^{YD}$ mice resulted in a decreased time to tumor development and the development of metastases within the lung parenchyma (extravascular metastases). It was further shown in this study that expression of a dominant active TβRI (MMTV-TβRI$^{AAD}$) was also able to influence mammary development and tumorigenesis. The TβRI$^{AAD}$ construct has 3 mutations: T204D to activate the kinase and L193A and P194A, which prevent inhibition of TβR1 by the ligand FKBP-12. The mice were unable to lactate properly as a result of increased rates of apoptosis within the mammary epithelium and decreased rates of proliferation. These mice were also crossed with the MMTV-c-*Neu*$^{YB}$ and MMTV-c-*Neu*$^{YD}$ mice. In both crosses the tumors took longer to develop compared with controls, but these mice had an increased incidence of lung metastases. The results using the TβRI$^{AAD}$ construct were similar to those using the constitutively active Alk5$^{T204D}$ (Oft et al., 1998). In the MMTV-Alk5$^{T204D}$ transgenic mouse model spontaneous tumors did not occur, however when crossed with MMTV-Neu mice the bigenic mice had an increased incidence of metastases compared with the MMTV–*Neu* mice. Systemic inhibition of TGF-β1 using the chimeric IgG–TβRII fusion protein (Fc–TβRII) also reduced tumor cell viability, migration, intravasation and lung metastasis in the MMTV-PyVmT transgenic mouse model, and mice transplanted with 4T1 or EMT6 mammary tumor cells (Muraoka et al., 2002). Together the results from transgenic TGF-β, dnTβRII, TβRII$^{\Delta Cyt}$, TβRI$^{AAD}$ and Alk5$^{T240D}$ expression during development and tumorigenesis further indicate that TGF-β signaling can act as an early tumor suppressor, but can later enhance tumor progression (Table 6).

**Table 6. Mouse models used to study mammary development and tumorigenesis.** Many of the models use a mouse mammary tumor virus (MMTV) promoter/enhancer to drive expression of transgenes in the mammary gland epithelium throughout development, pregnancy, lactation and involution. The whey acidic protein (WAP) promoter drives expression of transgenes in the mammary lobular-alveolar epithelium around day 13 of pregnancy through the second day of post-lactation involution. The beta-lactoglobulin (Blg) promoter drives expression primarily in secretory lobular-alveolar epithelium. In the mammary gland the use of a zinc inducible metallothionine (MT) promoter resulted in transgene expression primarily in the fibroblast population. TGF-β1s$^{223/225}$, TGF-β3, TβRI (T204D) and TβRI (AAD) activated, while dominant negative TGF-β type II receptor (dnTβRII) and the chimeric IgG/TβRII fusion protein (Fc:TbetaRII) attenuated the TGF-β pathway *in vivo*. In the conditional *Tgfbr2* mouse model, expression of Cre in mammary epithelium, mediated recombination of LoxP sites flanking exon 2 of the *Tgfbr2* gene, resulting in excision of the flanked region. In addition to chemical carcinogenesis, tumors occurred spontaneously or were induced through expression of transforming growth factor alpha (TGF-α), polyoma middle T antigen (PyVmT) or ErbB2 (neu) as indicated.

**Table 6. Mouse models used to study mammary development and tumorigenesis.**

| Model | Promoter | Transgene | Tumour Induction Strategy | Details |
|---|---|---|---|---|
| Transgenic | MMTV | TGF-β1$^{s223/225}$ | | Hypoplasia of mammary ducts with alveolar outgrowths that were able to produce enough milk to sustain full litters. |
| Transgenic | WAP | TGF-β1$^{s223/225}$ | | Inhibition of lobular-alveolar structures and milk protein expression. This phenotype was also present in transplanted glands. Premature aging of the mammary epithelial cells thought to be the result of stem cell senescence. |
| Transgenic | MMTV | TGF-β1$^{s223/225}$ | MMTV-TGF-α DMBA | Latency was increased in the TGF-β1/TGF-α model, and the TGF-β1 transgenic mice were also resistant to 7,12-dimethylbenz[a]anthracene-induced mammary tumours. |
| Transgenic | MT | dnTβRII | | Expression of a dominant negative TβRII (dnTβRII) under control of a zinc inducible metallothionine derived promoter resulted in expression mainly located in the mammary stroma. Abrogation of TβRII signaling in the stroma was associated with increased HGF expression and lateral branching in the adjacent epithelium. |
| Transgenic | Blg | TGF-β3 | | Increased apoptosis of mammary epithelium during involution. |
| Transplanted Homozygous Null *TGF-β 3* | *TGF-β 3* | | | Inhibition of apoptosis was observed in mammary epithelium during involution. Homozygous null transplants were compared to *TGF-β 3* +/- controls to determine statistical significance. |
| Transgenic | WAP | TGF-β1$^{s223/225}$ | MMTV Injection | TGF-β1 expression correlated with a significant resistance to tumourigenesis induced by the mouse mammary tumour virus (MMTV). The number of preneoplastic cells was shown to be decreased in TGF-β1 transgenic mice compared to the controls. |
| Heterozygous Null *TGF-β 1* | *TGF-β 1* | | | Ovariectomized mice had no phenotype, but demonstrated a 10-15 fold increase in proliferation over the wild type mice when both populations were supplemented with estrogen and progesterone. Hyperproliferation was observed in mammary epithelium and this was shown to be a cell autonomous effect. |
| Transgenic | MMTV | Fc:TbetaRII | MMTV-c-neu and tail vein injection of tumour cells | Significant levels of Fc:TbetaRII were detected in the serum and most tissues. Mice were resistant to metastasis in the MMTV-c-neu background or upon injection of isogenic malignant melanoma cells through the tail vein. Importantly, no adverse effects due to prolonged expression of the TGF-β inhibitor were noted. |
| Transgenic | MMTV | dnTβRII | MMTV-TGF-α | Expression of the dominant negative TβRII transgene (dnTβRII) resulted in alveolar hyperplasia and differentiation in virgin mice, precocious differentiation during early pregnancy while development during late pregnancy and lactation was impaired. In this transgenic line involution was also delayed. When crossed with the MMTV-TGF-α line, tumour latency and invasion were decreased. Invasive lesions demonstrated a suppression of dnTβRII. Spontaneous tumours were observed around 27.5 months in the dnTβRII animals. |
| Transgenic | MMTV | TβRII antisense | | Full length antisense RNA expression resulted in precocious lobular-alveolar development. |
| Transgenic | MMTV | TGF-β1$^{s223/225}$ | MMTV-c-neu | No difference in tumour latency. Bigenic tumours expressed more activated Smad2, p38 MAPK, Akt and Rac1. Bigenic tumours also demonstrated higher levels of vimentin. In addition the bigenic tumours were more invasive and metastatic than the MMTV-c-neu controls. |
| Transgenic | MMTV | dnTβRII | MMTV-neu YB MMTV-neu YD | This dominant negative TβRII model (TBRIIΔCyt) did not exhibit a developmental phenotype. The modified neu transgenes couple constitutive Grb2 (YB) and Shc (YD) activation. During tumourigenesis this model resulted in a decreased latency and extravascular metastasis to the lung. |
| Transgenic | MMTV | TβRI (AAD) | MMTV-neu YB MMTV-neu YD | This dominant active TβRI transgene has 3 mutations: T204D to activate the kinase and L193A/P194A that prevent inhibition by FKBP-12. The observed developmental lactation deficiency was attributed to increased apoptosis decreased proliferation. The modified neu transgenes couple constitutive Grb2 (YB) and Shc (YD) activation. In this model an increased latency was observed in both tumour models with enhanced extravascular metastasis to the lung. |
| Transgenic | Inducible MMTV | TGF-β1$^{s223/225}$ | MMTV-PyVmT | Two weeks of TGF-β induction in MMTV-PyVmT tumours was sufficient for >10 fold increase in the number of lung metastases. No effect was observed in primary tumour size or cell proliferation upon TGF-β induction. |
| Conditional Null *Tgfbr2* | *Tgfbr2* | MMTV-Cre | MMTV-PyVmT | Lobular-alveolar hyperplasia and increased apoptosis. Tumour latency was decreased while the number of metastases increased when crossed with MMTV-PyVmT mice. |
| Transgenic | MMTV | TβRI (T204D) | MMTV-c-neu | Expression of the constitutively active TβRI (T204D) reduced apoptosis in the terminal endbuds and during post-lactational involution. Expression of TβRI (T204D) in the presence of MMTV-c-neu increased metastasis. |

**Attenuation *versus* complete mammary specific deletion of TβRII signaling during development and tumorigenesis**

During mammary development, deletion of the loxP flanked *exon 2* from *Tgfbr2* using MMTV-Cre mediated recombination resulted in lobular-alveolar hyperplasia accompanied by an increase in the rate of apoptosis (Forrester et al., 2005), much like the results from the expression of a dominant negative TβRII transgene, TβRII antisense or loss of a *Tgfβ1* allele in the mammary gland (Ewan et al., 2002; Gorska et al., 2003; Lenferink et al., 2003) This result indicated that TGF-β signaling during development in the mammary epithelium may function to limit growth while concurrently acting as a cell survival signal. It was previously shown that the response to systemic hormones could be increased by the reduction of TGF-β signaling in the mammary epithelium (Ewan et al., 2005; Ewan et al., 2002), however this link has not been tested at present in the TβRII conditional null mouse model. When conditional MMTV-Cre TβRII mice were crossed with the MMTV-PyVmT mouse line, mammary tumors developed more rapidly and an increase in the numbers of lung metastases was observed (Forrester et al., 2005). This model was the first to demonstrate the effect of complete ablation of endogenous TβRII expression in the context of cell autonomous tumor initiation and progression *in vivo*. The results obtained in the absence of TβRII were interesting, since they appeared contradictory to the previous results discussed above--that TGF-β is a suppressive factor during early tumorigenesis, but later it increases tumor progression and metastasis (Gorska et al., 2003; Muraoka-Cook et al., 2004; Muraoka-Cook et al., 2005b; Oft et al., 1998; Siegel et al., 2003; Tang et al., 2003). Furthermore, it had been hypothesized that TGF-β signaling was necessary for invasion and metastasis to occur through the induction of an epithelial-mesenchymal transition (EMT) *in vivo* (Deckers et al., 2006;

Oft et al., 1998). It is now clear that there are differences between reducing the TGF-β signal in a tumor cell through mechanisms such as dominant negative receptor expression and genetic modifications that lead to a complete loss of TβRII expression. Together, observations from these pivotal mouse studies, both new and old, indicate that the presence and relative level of TGF-β pathway activation together regulate cancer cell behavior *in vivo*.

**Lessons related to the dual role for TGF-β regulation of tumorigenesis from studies using skin carcinogenesis as a model system**

In a defining set of experiments, TGF-β was clearly shown to be both a tumor suppressor and tumor promoter *in vivo* in five different mouse models of skin carcinogenesis (Cui et al., 1996). Mice expressing a TGF-β transgene targeted to the suprabasal keratinocytes in the skin (four TPA inducible transgenic lines and one constitutive line (Cui et al., 1995; Fowlis et al., 1996)), showed that expression of TGF-β1 was able to initially suppress the growth of benign DMBA/TPA induced tumors and then to increase the malignant conversion rates of the benign papillomas, the rate of tumor progression (Cui et al., 1996). Interestingly, endogenous expression of TGF-β1 and TGF-β3 was increased in the spindle cell carcinomas (Cui et al., 1996). This study was later complemented by inhibition of TGF-β signaling through tissue specific transgenic expression of a dominant negative TβRII in mouse epidermal basal and follicular cells (Amendt et al., 1998). Expression of this transgene did not disturb normal homeostasis. However, attenuation of TGF-β signaling in the basal and follicular cells did enhance carcinogenesis, with a 25% increase in the conversion frequency from

papilloma to carcinoma, during TPA induced tumor promotion after initiation by DMBA (Amendt et al., 1998). In this study, it was also reported that carcinomas occurred more frequently in transgenic mice after DMBA induced initiation without administration of a tumor promoter such as TPA. Correlating with these results, chronic TPA administration was shown to increase the rate of proliferation in the epidermis of transgenic mice expressing the dominant negative TβRII transgene (Amendt et al., 1998).

Similar studies later confirmed and extended these initial observations (Go et al., 2000; Go et al., 1999). Inducible expression of TGF-β1 in mice, under the control of the epidermal interfollicular keratinocyte specific loricrin promoter, demonstrated that early expression resulted in tumor suppression and induction at later stages promoted a rapid progression to metastasis (Wang et al., 1999; Weeks et al., 2001). In this system a reduction in the expression level of Smad2, Smad3, Smad4, Alk-5 and TβRII was observed in the DMBA/TPA induced papillomas from the TGF-β1 expressing transgenic mice, but it was not possible to determine if this reflected normal events during tumor progression or artifacts due to prolonged transgenic TGF-β1 expression (Han et al., 2005; Weeks et al., 2001). These results correlate with the pattern of TGF-β expression in human skin cancer samples: immunohistochemistry showed that increased expression of TGF-β1 was evident and suppression of TβRII expression correlated with tumor progression (Han et al., 2005). In order to address issues related to concurrent cell autonomous induction of TGF-β1 expression and the downregulation of components from the TGF-β signaling pathway in skin tumorigenesis, the same TGF-β1 transgenic mouse line was crossed with a loricrin promoter driven dnTβRII mouse line (Han et al., 2005; Wang et al., 1997).

**Table 7. Mouse models used to study skin development and tumorigenesis.** Many of the mouse models designed to study the effects of TGF-β in the skin have used the keratin 6 and keratin 10 promoters to target suprabasal keratinocytes or the loricrin promoter to drive transgene expression in the interfollicular epidermis. In addition, the keratin 5 promoter has been used to drive transgene expression in the basal cell compartment and interfollicular epidermis and outer root sheath cells of the hair follicle in the skin. *Inducible expression dependent upon hyperplasia achieved through TPA administration. ** Inducible expression of TGF-β1 $^{s223/225}$ was achieved through transgenic expression of a loricrin promoter driving the GLVPc transactivator (a fusion molecule of the truncated progesterone receptor and the GAL4 DNA binding domain), and a thymidine kinase promoter with GAL4 binding sites upstream of the promoter driving TGF-β1$^{s223/225}$ expression. TGF-β1 $^{s223/225}$ expression activated, while dominant negative TGF-β type II receptor (dnTβRII) attenuated the TGF-β pathway *in vivo*. DMBA/TPA is a common two-step chemical carcinoma induction protocol applied to the skin wherein DMBA initiates and TPA promotes tumorigenesis.

## Table 7. Mouse models used to study skin development and tumorigenesis.

| Model | Promoter | Transgene | Tumour Induction Strategy | Details |
|---|---|---|---|---|
| Transgenic | *Keratin 6 | mTGF-β1 TGF-β1$^{s223/225}$ | | Inducible expression in suprabasal keratinocytes from the skin. The TGF-β encoded in the mTGF-β1 transgenic constructs (D and F lines) encoded the native latent murine form of the protein. Alternatively, the TGF-β1$^{s223/22}$ transgenic lines (M2 and M5) produced a constitutively active TGF-β protein. The keratin 6 promoter in this context was predominantly active in the hyperplastic state and therefore inducible through TPA administration. Keratinocytes were also shown to express under this promoter when cells were isolated and placed into cell culture conditions. |
| Transgenic | Keratin 10 | TGF-β1$^{s223/225}$ | | Targets suprabasal keratinocytes in the skin constitutively. Interestingly, the DNA labeling index in transgenic mice was increased without signs of hyperplasia. TGF-β was shown to act as a negative growth regulator when challenged with TPA. |
| Transgenic | Keratin 10 *Keratin 6 | mTGF-β1 TGF-β1$^{s223/225}$ | DMBA/TPA | This study was completed using five transgenic mouse lines: the four Keratin 6 driven lines previously described (D, F, M2 and M5) and the Keratin 10 driven TGF-β1 H line also previously described. TGF-β1 inhibited benign DMBA/TPA induced tumour outgrowth while increasing tumour progression and spindle cell conversion rate. TGF-β also increased the benign papilloma malignant conversion rates. Further, endogenous TGF-β1 and TGF-β3 were upregulated in the associated spindle cell carcinomas. This was the first study to clearly demonstrate that TGF-β could function as both a tumour suppressor and a tumour promoter in vivo. |
| Transgenic | Loricrin | dnTβRII | | Heterozygous transgenics: Thickened, wrinkled, hyperplastic and hyperkeratotic epidermis; Increased proliferation in basal and suprabasal epidermis; At 10-14 days the transgenics were indistinguishable from the controls. Homozygous transgenics: Perinatal lethality associated with restricted movement due to severe hyperkeratotic phenotype. Cultured primary keratinocytes: More proliferative in vitro. |
| Transgenic | Keratin 5 | dnTβRII | DMBA/TPA | No developmental phenotype. Hyperproliferation during tumour promotion. Increased malignant conversion frequency from benign papillomas to carcinomas during tumourigenesis. Increase in carcinomas without tumour promotion. |
| Transgenic | **Loricrin | TGF-β1$^{s223/225}$ | TPA | Generation of "gene-switch" mice that permit inducible expression of TGF-β1. Decrease in epidermal proliferation. Hyperplasia associated with phorbol 12-myristate 13-acetate (PMA also known as TPA) induction, accompanied by a decrease in epidermal thickness and proliferation. Increased dermal angiogenesis. |
| Transgenic | Loricrin | dnTβRII | DMBA/TPA | Thickened skin due to epidermal hyperproliferation. Shorter papilloma latency and increased tumour number in transgenic mice. Rather than regression of papillomas, as observed in control mice, the transgenic papillomas progressed to carcinomas. TPA alone caused papillomas in transgenic mice suggesting loss of TGF-β signaling contributed to the initiation of tumourigenesis. Transgenics demonstrated an increase in neovascularization and progressed to metastasis while histologically the tumours appeared to be carcinoma in situ or well differentiated. VEGF and TGF-β1 levels increased, while TSP-1 levels decreased in the transgenic mice, corresponding with the increase in neovasculature. |
| Transgenic | Loricrin | dnTβRII | DMBA/TPA | Increased susceptibility to chemically induced carcinogenesis at early and late stages. Transgenic papillomas had an increase in S-phase by flow cytometry and proliferation quantified by analysis of BrdU incorporation and mitotic indexes. BrdU and S-phase indexes were not significantly different from the controls when the tumours progressed to squamous cell carcinoma (SCC). Transgenic SCCs had and increase in mitotic indexes and G2/M as quantified by flow cytometry. Transgenic tumours also exhibited a decrease in p15(INK4b), p21 (WAF-1) and p27 (Kip1) expression. |
| Transgenic | **Loricrin | TGF-β1$^{s223/225}$ | DMBA/TPA | Using an inducible loricrin promoter it was shown that TGF-β1 expression early in tumourigenesis was tumour suppressive, however in papillomas expression rapidly induced metastasis. Transgenic tumours demonstrated an increase in angiogenesis corresponding with a decrease in TSP-1 and TSP-2 expression. Transgenic tumours also demonstrated higher levels of MMP-2 and MMP-9. During progression, TβRI, TβRII, Smad2, Smad3 and Smad4 were downregulated. The E-cadherin complex was also lost in transgenic papillomas. These markers were only lost in malignant carcinomas from the control mice. |
| Transgenic | **Loricrin/ Loricrin | TGF-β1$^{s223/225}$ dnTβRII | DMBA/TPA | Inducible expression of TGF-β1 in epidermal keratinocytes expressing a dominant negative type II TGF-β receptor. Late stage TGF-β1 induction alone failed to inhibit tumour growth, but did increase metastasis and EMT resulting in spindle cell carcinoma (SPCC). The dnTβRII transgene abrogated the TGF-β1 induced EMT. The bigenic tumours (inducible TGF-β1 and dnTβRII) demonstrated a higher number of metastasis, in opposition to predictions based on retention of junction related protein localization. EMT activity was correlated with abrogation of Smad activation by dnTβRII. RhoA/Rac and MAPK induction by TGF-β1 in the presence of dnTβRII were not altered and thereby permitted their contribution to metastasis. EMT requires TβRII while invasion mediated by TGF-β1 acts synergistically with a reduction in the level of TβRII expression. |

Skin tumors were induced in these mice using DMBA and TPA. The highest occurrence of malignant tumors was observed in the TGF-β1;dnTβRII mice followed by the TGF-β1 transgenics, the dnTβRII transgenics and the control mice respectively. Interestingly, 30% of the TGF-β1 transgenic animals exhibited spindle cell carcinoma (SPCC), whereas lesions in the other genotypes were predominantly classified as squamous cell carcinoma. The SPCC lesions have a characteristic EMT morphology, and this correlates with loss of proper junctional adhesion protein localization at the cell membrane and increased expression of components of the Notch signaling pathway, Hey1 and Jag1. Conversely, the dnTβRII and TGF-β1;dnTβRII metastases retained junction related protein expression at the cell membrane, but despite this, TGF-β1;dnTβRII mice had the highest rate of metastasis (Han et al., 2005). These results clearly demonstrate that in the skin, TGF-β is an early tumor suppressor, and tumor cell autonomous loss of TGF-β response accompanied by an increase in TGF-β production can promote tumor progression (Table 7).

**Fibroblast associated signaling in the tumor microenvironment**

TGF-β mediated signaling has been shown to regulate epithelial and fibroblast cell autonomous functions during the past two decades, but recently we are beginning to appreciate the magnitude of downstream stromal-epithelial interactions that contribute to the regulation of cancer. Many of the early studies, and pioneering work in the field of stromal-epithelial interactions, have been able to demonstrate clear interactions between the stromal and epithelial compartments during tumorigenesis. An illustration for the

general stromal-epithelial interactions involved in the regulation of cancer is clearly shown when benign prostatic hyperplasia epithelial cells (BPH-1) are grafted under the mouse kidney capsule with carcinoma associated fibroblasts (CAFs) (Hayward et al., 2001). Further, the malignant transformation of the epithelial cells by the CAFs resulted in permanent changes in the epithelial cell population. The epithelial cells cultured from tumors in which BPH-1 and CAF cells had been mixed, were able form colonies in soft agar whereas the controls did not acquire this trait (Hayward et al., 2001). A more recent study using human derived CAFs clearly outlined some of the major characteristics for these cells and their contribution to adjacent tumor growth (Orimo et al., 2005). Fibroblasts extracted from invasive human breast cancers potentiate tumor growth more effectively than those isolated from outside the tumor mass. The fibroblasts isolated from within the tumor masses also expressed α-SMA and exhibited an increase in contractility which indicates that the majority of these cells may be myofibroblasts. The CAFs were able to promote vascularization, and SDF-1 produced by the fibroblasts contributed to the recruitment of endothelial progenitor cells in the tumor mass. Further, the SDF-1 produced by the CAFs contributed to an increase in growth through the cognate CXCR4 receptor expressed by the human breast carcinoma cells (Orimo et al., 2005). Previous experiments profiling gene expression through SAGE analysis resulted in identification of several other genes expressed by myofibroblasts including *CXCL12* that promote tumor growth (Allinen et al., 2004). The observed *CXCL12* expression was verified as a myofibroblast limited gene product through mRNA in situ hybridization. It was shown that CXCL12 was able to induce proliferation of MDA-MD-231 and MCF10A cells. The expression of CXCL12 was also able to induce an increase in migration and invasion of

the MDA-MB-231 cells (Allinen et al., 2004). It is clear that stromal fibroblasts and myofibroblast cells within the tumor microenvironment have an impact on tumor progression.

Advances in stromal-epithelial signaling related research have been instrumental in the analysis of interactions regulating the tumor microenvironment. The stroma in the mammary gland has been shown to regulate the tumorigenesis in adjacent epithelial cells when modified *in vivo*. Several key studies involving breast cancer have recently addressed the issue of stromal-epithelial interactions and the data suggests that stromal fibroblasts may act as mediators of the adjacent epithelial cell compartment to regulate tumor initiation and progression. Irradiation of mouse mammary fat pads, can provide an activated stromal compartment that promotes tumorigenesis of otherwise non-tumorigenic epithelial cells (Barcellos-Hoff and Ravani, 2000). In this study the host mammary fat pads were cleared and irradiated to produce an activated stroma in which COMMA-D epithelial cells were implanted. The COMMA-D line harbors genetic mutations including the loss of *p53* on both alleles, but it does not consistently form tumors when implanted into wild type syngeneic non-irradiated control mice. In the mice that were treated by irradiating the cleared mammary fat pads, introduction of this epithelial cell line at three days post irradiation produced a four-fold increase in tumor incidence at six weeks post implantation. In addition to an increase in incidence within irradiated stromal implants the results indicated an increase in size and rate of tumor growth as compared to the controls. The irradiation in these experiments limited systemic contributions to tumor progression though hemibody delivery. This further suggested that local stromal influences were responsible for the observed changes in

tumorigenesis since the control and irradiated tissues were implanted within the same mouse (Barcellos-Hoff and Ravani, 2000).

Another important illustration of the precedence for stromal-epithelial interactions in cancer research came from a study which, in addition to demonstrating stromal-epithelial interactions, developed a novel system wherein human stromal cells could be engineered into the mouse mammary fat pad thereby allowing introduction of human epithelial cells *in vivo* (Kuperwasser et al., 2004). Human epithelial cells do not normally reconstitute the mouse mammary gland when introduced into clear mammary fat pads due to the lack of signals from human mammary fibroblasts that are necessary for their function *in vivo*. This limitation had previously made it difficult to determine if human epithelial cells had the same dependence on fibroblast regulation since well controlled experiments could not be adequately constructed to model this interaction. In the study by Kuperwasser and colleagues they were able to circumvent this problem by introducing irradiated human fibroblasts admixed with non-irradiated normal fibroblasts into the mouse mammary fat pad. The introduction of the admixed fibroblasts resulted in colonization by the human fibroblasts in a process they refer to as humanization of the mouse mammary fat pad. Human epithelial cell organoids (myoepithelial and luminal epithelial cell clusters) derived from reduction mammoplasty in patients aged 29-37 years old were able to colonize the humanized mouse mammary fat pad when mixed with human fibroblasts prior to implantation. The human epithelial cell colonization resulted in growth of normal ductal tissue, benign hyperplastic ducts (hyperplasia), in situ ductal cancer (DCIS), and invasive carcinoma. The resulting tissues, exhibiting various hyperplastic and neoplastic lesions, were interesting since the samples used from

reduction mammoplasty were relatively normal prior to implantation (Kuperwasser et al., 2004). These experiments suggest that in human, as reflected in mouse cancer models, stromal signaling can regulate adjacent epithelial cell tumorigenesis.

**Fibroblast to myofibroblast transdifferentiation and tumor progression**

One likely explanation for our observed increase in smooth muscle actin positive stromal fibrovasculature is that the fibroblasts are induced to become myofibroblasts. It has been shown that some fibroblasts that respond to TGF-β have the potential to transdifferentiate into a myofibroblast cell type. In light of recent evidence demonstrating elevated stromal TGF-β1 expression in tumors with TβRII deficient carcinoma cells (Yang et al., 2008), this is a likely mechanism for induction of a fibroblast to myofibroblast transdifferentiation process *in vivo*. It has been suggested that fibroblasts are at first attracted to low levels of TGF-β1 (Postlethwaite et al., 1987), but as they approach the tumor microenvironment the concentration of TGF-β increases leading to their transdifferentiation (De Wever and Mareel, 2003). In the tumor microenvironment myofibroblasts promote tumorigenesis through secretion of factors that are thought to contribute to tumor progression (Allinen et al., 2004; De Wever et al., 2004a; Orimo et al., 2005). It was recently shown that squamous cell carcinoma (SCC) cells can induce normal fibroblasts to transdifferentiate into myofibroblast cells. This study demonstrated that TGF-β1 produced by the SCC cells led to the transdifferentiation of the normal fibroblast cell population. Myofibroblast cells derived from the transdifferentiation process produced significantly higher levels of hepatocyte growth

factor (HGF) and promoted an increase in migration on matrigel (Lewis et al., 2004). In addition to the expression of TGF-β1 from carcinoma cells, TGF-β1 may be upregulated in the stromal cells of malignant tumors. The stromal upregulation of TGF-β is not a new concept and has been shown in a number of systems including human carcinoma associated fibroblasts derived from the prostate when compared to normal human prostatic fibroblast populations. Further, the TGF-β1 produced by the carcinoma associated fibroblasts was able to promote an increase in colony formation on soft agar (San Francisco et al., 2004).

The process of myofibroblast transdifferentiation in response to TGF-β1 expression is not strictly limited to tumor associated fibroblasts. An interesting demonstration of this process was performed using PS-1 prostate fibroblast cells. In the PS-1 cells both androgen and TGF-β1 were able to induce myofibroblast transdifferentiation alone or in combination. Upon stimulation with TGF-β1 the PS-1 cells demonstrated a translocation of the androgen receptor from the nucleus to cytoplasm during myo-transdifferentiation, and re-localization of the receptor to the nucleus after the transdifferentiation to a myofibroblast cell type was completed (Gerdes et al., 2004). Myofibroblasts have been shown in culture to lead invasion of colon cancer cells on a collagen matrix, and TGF-β production by the cancer cells was necessary for invasion. TGF-β1 was shown to be necessary and sufficient for invasion of myofibroblasts and induced N-cadherin expression at the tips of the filopodia. N-cadherin expression was necessary for invasion, and the upregulation by TGF-β1 was found to be induced by the jun N-terminal kinase (JNK), a known downstream target of TGF-β receptor activation (De Wever et al., 2004b). Another change in signaling that may be attributed to TGF-β1

induced transdifferentiation of fibroblasts to myofibroblasts, includes modulation and balance of RhoA/Rac activity. This was demonstrated along with the synergistic signaling of Tenascin C and HGF expressed by myofibroblasts upon adjacent human colon cancer cells (De Wever et al., 2004a).

Integrins may also play a role in TGF-β mediated fibroblast to myofibroblast transdifferentiation. The integrins αvβ5 and αvβ3, have been shown to contribute to myofibroblast transdifferentiation in fibroblasts derived from human mouth and skin, since this process was diminished when these elements were blocked. Fibroblasts derived from kidney in this study were only dependent on αvβ5, and this suggests that there may be differences in the requirements for these factors or alternate thresholds for signaling in these cell types (Lygoe et al., 2004). The association of αvβ3 integrin with TβRII using bioluminescence resonance energy transfer (BRET) has recently been demonstrated, and signaling through αvβ3 was shown to enhance TGF-β mediated growth stimulation in human lung fibroblasts. The interaction with TβRII was not observed with αvβ5 or α5β1 integrins (Scaffidi et al., 2004). These results suggest that the specific integrins have the ability to potentiate TGF-β, and it is likely that other integrin family members will demonstrate specific interactions involving fibroblast signaling in this pathway when they are examined experimentally.

Recently, a characterization of gene expression during the transdifferentiation process has been performed in human lung fibroblasts after stimulation with TGF-β (Chambers et al., 2003). Timepoints used for analysis were 1.5, 6, 16 and 24 hours after treatment. Many of the upregulated genes demonstrated a progressive increase in expression over the timecourse of the experiment. Genes

identified during the timecourse included subgroups of cytoskeletal reorganization, matrix formation, metabolism and protein synthesis, cell signaling, proliferation and survival, gene transcription and a group of genes that do not fit into any of these categories. The fibroblast cells before treatment expressed low levels of smooth muscle α-actin and h-caldesmon. In addition, prior to treatment the cells were vimentin positive and desmin negative. When TGF-β was used for stimulation of the fibroblasts for 36 hours, in agreement with the array data, there was a marked increase in smooth muscle α-actin, h-caldesmon and smooth muscle myosin heavy chain. Further, by immunocytofluorescent microscopy smooth muscle α-actin, h-caldesmon and smooth muscle myosin heavy chain were shown to be organized in fibers and filaments indicating proper assembly after TGF-β stimulation (Chambers et al., 2003).

Currently, there is sufficient evidence to suggest that TGF-β mediated fibroblast to myofibroblast transdifferentiation can contribute to tumor progression. Our current results suggest that loss of carcinoma cell specific TGF-β signaling can lead to the recruitment, transdifferentiation or selection of adjacent smooth muscle actin positive fibroblasts *in vivo* (Bierie et al., 2008) and Chapter III. If transdifferentiation is responsible for the phenotype then enhanced TGF-β production, which we have observed (Yang et al., 2008), within the TGF-β signaling deficient mammary tumor microenvironment may be partially responsible for this phenotype. Regardless of the mechanism whereby the stroma is altered, the important part is that there are more SMA+ fibroblasts in the tumors with a carcinoma cell specific TGF-β signaling deficiency and this cell population has been linked to increased carcinoma cell invasion and metastasis.

**TGF-β mediated stromal-epithelial interactions in the tumor microenvironment**

The results that we have attained suggested that one of the contributions to the smooth muscle actin expression in fibroblast cells from tumors with a carcinoma cell specific TGF-β signaling deficiency may be the increased TGF-β production present in those tissues (Bierie et al., 2008; Yang et al., 2008) and Chapter III. Therefore, it is important to consider the known roles for TGF-β in this adjacent cell population. Fibroblast contributions to cancer initiation and progression have been the subject of recent investigation with respect to TGF-β mediated regulation of cancer. Notably, the current studies addressing stromal fibroblast regulation of proliferation in adjacent epithelial cells can be partially attributed to initial work performed over two decades ago (Cunha et al., 1980). At present, there is not a model of activated TGF-β signaling that specifically targets the fibroblast cell population. However, several key observations in TGF-β signaling deficient fibroblasts have offered strong evidence to promote the concept that fibroblasts can significantly regulate adjacent epithelial or carcinoma cell populations *in vivo*.

Stromal-epithelial interactions can mediate adjacent epithelial cells leading to both developmental defects and carcinoma if sufficiently altered *in vivo*. Ablation of *Tgfbr2* in stromal fibroblasts resulted in developmental defects and carcinoma of adjacent epithelial cell populations (Bhowmick et al., 2004a; Cheng et al., 2005; Cheng et al., 2007; Chytil et al., 2002). In the mammary gland *Tgfbr2* was ablated from the fibroblast cell population and this resulted in a decrease of proliferation in the terminal end buds accompanied by an increase in apoptosis from the ductal cell compartment (Cheng et al., 2005). In the same mouse model it was found that ablation of *Tgfbr2* in stromal

fibroblasts can lead to prostatic intraepithelial neoplasia and invasive squamous cell carcinoma of the forestomach (Bhowmick et al., 2004a). In the case of forestomach and prostate, p27 and p21 were downregulated in the TβRII null tissues while conversely c-myc was upregulated. Further, in the forestomach both c-met phosphorylation and pro-HGF were upregulated indicating a possible contribution to the observed occurrence of squamous cell carcinoma. The HGF ligand was also found to be expressed in the prostate fibroblasts (Bhowmick et al., 2004a). The consistency in HGF ligand expression suggests that this TGF-β dependent mechanism can contribute to the regulation of carcinoma progression.

Experiments conducted in fibroblasts derived from a mouse model in which TβRII was conditionally ablated were informative in illustrating several mechanisms that allow the fibroblast cell population to influence adjacent epithelial cells (Cheng et al., 2005). In xenograft experiments under the kidney capsule, TβRII null mammary fibroblasts implanted with mammary carcinoma cells were able to cause an increase in phosphorylation of erbB1, erbB2, RON and c-met when compared to the controls. The increased phosphorylation for these central signaling receptors also correlated with an increase in tumor growth and invasion. Expression of TGF-α, MSP and HGF were also shown to be upregulated by fibroblasts in this study, further suggesting that many potent epithelial cell signaling components were involved in the TGF-β mediated fibroblast specific stromal-epithelial regulation of cancer. Treatment of tumor cells with conditioned medium from TβRII null fibroblasts lead to an increase in proliferation and motility. In addition, blocking TGF-α using pharmacological inhibitors or MSP, HGF and c-met by neutralizing antibodies prevented the stimulation observed in the un-treated

controls (Cheng et al., 2005). These experiments provide a novel mechanism wherein TGF-β signaling suppresses expression of TGF-α, MSP and HGF signaling that normally function to enhance progression in the tumor microenvironment.

The regulation of cancer initiation, progression and metastasis involves the entire cast of players in the tumor microenvironment including both resident and transient cell populations. Cancers are often composed of cells that harbor alterations in a number of pathways through genetic mutation or epigenetic regulation of expression. Many of the predominant pathways altered in human cancer have been analyzed to determine their relative contribution to TGF-β mediated regulation of tumorigenesis. Recently it has been shown that expression of HER2 unmasks the migratory response to TGF-β in epithelial cells (Ueda et al., 2004). The regulation of migration was shown to be dependent upon signaling through the PI3K, MAPK, P38 MAPK and Integrin-β1 which are often present and active in many cancers. In addition to migration and invasion, expression of EGF and HGF can inhibit TGF-β dependent growth inhibition (Massague and Chen, 2000). Experimental evidence has also shown that activation of EGF, HGF, Erk or Ras signaling can inhibit activity of the central signaling components, Smads 2 and 3 (Calonge and Massague, 1999). These results further suggest that the erbB and HGF signaling regulated by TGF-β in stromal fibroblasts can have an impact on the growth and migration of adjacent epithelial cell populations *in vivo*. Together, the data clearly demonstrates that signals derived from fibroblasts can have an impact on adjacent normal and carcinoma associated mammary epithelium. Since we have observed differences in both the abundance and phenotype of stromal fibroblasts within our tumors with a carcinoma cell specific ablation of TGF-β signaling, it is likely that the alternate

fibroblast cell populations regulate tumor progression in different ways. It would be informative to isolate the fibroblast cells associated with the two types of tumor tissue and determine how they individually contribute to the regulation of tumor progression and metastasis. However, the myofibroblast-like phenotype exhibited by the stroma in the tumors with TGF-β signaling deficient carcinoma cells suggests that they promote these processes *in vivo*.

**Carcinoma cell mediated selection of stromal fibroblasts within the tumor microenvironment**

Carcinomas contain not only transformed epithelial cells, but also stromal fibroblasts that may be altered or activated. Our data suggested that loss of TGF-β signaling in mammary carcinoma cells may select for a smooth muscle positive fibroblast cell population *in vivo*. This parallels results described in other systems wherein, it has been proposed that the epithelial cells influence the identity and behavior of adjacent fibroblasts in a tumor. Some convincing studies have been performed using the prostate as a model, in which the stromal-epithelial interactions have been well established. To demonstrate the interaction, modified stromal-epithelial interactions were demonstrated by recombining benign prostate hyperplasia-derived epithelial cells with CAFs (Hayward et al., 2001; Olumi et al., 1999). In this model, permanent changes were induced in epithelial cells by the CAFs that resulted in the formation of aggressive carcinomas (Hayward et al., 2001; Olumi et al., 1999). Importantly, at present it is not known precisely how the carcinoma cells cause permanent alterations in the adjacent stroma. However, one option for acquisition of a permanent alteration is the selection and

expansion of tumor promoting sub-populations of fibroblasts by adjacent carcinoma epithelium.

Tumor associated loss of heterozygosity (LOH), genetic mutation and epigenetic changes often occur in both tumor cells and adjacent cell populations during tumor progression. In a recent study, it was demonstrated that an epithelial cell cancer (carcinoma) in the murine prostate could initiate and promote expansion of stromal fibroblasts lacking p53, and subsequently result in the selection for highly proliferative tumor promoting carcinoma associated fibroblasts *in vivo* (Hill et al., 2005). Over the years it has been clearly shown that mutations and LOH occur in both the epithelium and stroma from the tumor microenvironment, but until now a direct link has not been established between these aberrations and resulting selective pressures derived from stromal-epithelial interactions. However, in a mouse model of prostate cancer, that elicits a paracrine *p53* mediated downregulation of fibroblast proliferation, the selective expansion of a *p53*-/- fibroblast cell population has now been demonstrated (Hill et al., 2005). This newly demonstrated presence of selective pressure from the carcinoma upon fibroblasts within the tumor microenvironment helps to explain the mutation frequency previously noted for *p53* in tumor associated stroma. It now appears that rather than actively inducing widespread *p53* mutation in tumor associated stromal fibroblasts, there is a selective evolution of a highly proliferative *p53* null sub-population of carcinoma associated fibroblasts. At present it is not known whether this phenomenon occurs in all tumor microenvironments or in response to other signaling aberrations. However, it is clear that carcinoma cells can select for alternate stromal cell populations that ultimately lead to a growth advantage during tumor progression.

Importantly, in human cancer, stromal mutation including loss of heterozygosity (LOH) in the genome is a common feature associated with tumor progression. Interestingly, analysis of the genome from 134 sporadic invasive breast carcinomas revealed that the stromal components exhibited a higher number genes preferentially lost due to LOH as compared to the number lost due to LOH in the epithelium (Fukino et al., 2004). In this study it was reported 38 markers could be identified as preferentially lost in the stroma while 19 markers were preferentially lost in the epithelium. Since many of the markers, representing hot spots for mutation, in the stroma were non-redundant when compared to those identified in the epithelium it is likely that the stromal mutations were a result of independent mutation rather than divergence from a common progenitor (i.e., through epithelial to mesenchymal transition). Another interesting result obtained from this study, indicated that on average there was an increased frequency of LOH in the epithelium when compared to the stroma with the exception of three genes in which LOH occurred at a higher frequency in the tumor stroma. Genomic instability and mutation of individual cell populations in the microenvironment is likely a regulated process. Evidence for the regulation of mutation comes from the statistical analysis of genomic markers and direct analysis of the deleted regions from each cell population. Mutations observed at the genomic *11q* region are a good illustration of differentially regulated LOH in the genome. Six LOH hot spot markers were identified in the *11q* region with a 44Mb region deleted in the epithelium flanked by 29Mb and 11Mb regions deleted in the stroma. The 44Mbp region deleted in the epithelium notably contains the matrix metalloproteinase (MMP) gene cluster (Fukino et al., 2004). The stage of tumor progression and proximal distance from the primary tumor also impact the number of

LOH events in the stroma (Moinfar et al., 2000). Stromal components from human breast ductal carcinoma in situ (DCIS; n=7) exhibit fewer mutations at 10-65% of 12 markers selected for analysis than stromal components within infiltrating ductal carcinoma (IDC; n=5) that contained a 20-75% mutation rate. At a distance, 10 of the 12 markers displayed a 11-57% LOH rate indicating an association of mutation with the relative proximity to a tumor mass (Moinfar et al., 2000). Together these results indicate that LOH in the tumor microenvironment is regulated in a cell specific manner and the rate of LOH depends on the stage of tumor progression and proximity to the tumor mass.

Mutation and LOH represent two mechanisms wherein cell populations from the tumor microenvironment may be altered, however there are other genetic and epigenetic changes that can occur and these change clearly influence tumor progression. Epigenetic modification of gene expression is also dependent on the proximity to the tumor mass, stage of progression and cell type used for analysis (Hu et al., 2005). Using a new innovative technique called methylation-specific digital karyotyping these epigenetic trends were clearly demonstrated, and the results suggest that these epigenetic changes can alter gene expression thereby regulating disease progression (Hu et al., 2005). Genetic and epigenetic alterations are important since they either directly or indirectly influence gene expression profiles that ultimately regulate cancer initiation, progression and metastasis. In our tumor tissues, we have not performed analyses to determine if there are common genetic or epigenetic alterations in the stroma, however if the alternate fibroblast cell populations are selected rather than the result of transdifferentiation or differential recruitment these are two likely mechanisms that should be addressed.

**Bone marrow derived cells significantly contribute to tumor progression**

The tumor microenvironment is a complex mixture of cell types, matrix components and signaling molecules that provide a network of regulatory interactions. Our results demonstrated that a significant role for carcinoma cell specific response to TGF-β signaling is the suppression of chemokine expression and inflammation (Bierie et al., 2008; Yang et al., 2008) and Chapter III-IV. Specifically, we have shown that TGF-β potently suppresses the expression of *Cxcl1*, *Cxcl5* and *Ccl20* in mammary carcinoma cells and in non-transformed mammary epithelial cells (Bierie et al., 2008; Yang et al., 2008) and Chapter III-IV. Importantly, our laboratory has shown the Cxcr2, a receptor for both Cxcl1 and Cxcl5, is responsible for a significant portion of the bone marrow derived cell recruitment regulated by carcinoma cell specific response to TGF-β (Yang et al., 2008). We have been able to demonstrate the enhanced recruitment of an F4/80+ cell population to the tumor microenvironment when TGF-β signaling is ablated in mammary carcinoma cells. It is likely that this infiltrate represented both mature and immature bone marrow derived cell infiltrates. It has been known for many years that tumor associated macrophages can significantly contribute to tumor progression and metastasis (Coussens and Werb, 2002). However in recent years, the immature myeloid component has also been shown to significantly enhance tumor progression and metastasis. This immature cell population, termed myeloid immune suppressor cells (MISCs), is also known as myeloid derived suppressor cells (MDSCs). MISCs have been shown to represent approximately five percent of the cells within the tumor mass (Yang et al., 2004b). The MISC population is an immature myeloid derivative that expresses the GR1 and CD11b markers (GR1+CD11b+). Although this cell population was first described

over two decades ago and is now known to suppress the function of immune cells (Almand et al., 2001; Bronte et al., 2000; Gabrilovich et al., 1996; Kusmartsev and Gabrilovich, 2002; Kusmartsev and Gabrilovich, 2003; Serafini et al., 2004; Young and Lathers, 1999), the MISCs had not been directly associated with the regulation of tumor progression until recently (Yang et al., 2004b). The MISC cell population consists of immature macrophage, immature dendritic and hematopoietic progenitor cells (Almand et al., 2001; Yang et al., 2004b) that arise from altered host hematopoiesis induced by tumors (Almand et al., 2001; Bronte et al., 2000; Gabrilovich et al., 1996; Kusmartsev and Gabrilovich, 2002; Kusmartsev and Gabrilovich, 2003; Serafini et al., 2004; Young and Lathers, 1999) through currently unidentified systemic signals. In mice, the MISCs accumulate in the bone marrow, spleen and peripheral blood of tumor bearing hosts. MISCs have also been identified at a significant level in the peripheral blood of human tumor bearing hosts (Almand et al., 2001; Young and Lathers, 1999). One of the earliest studies to directly address the role for MISCs in the tumor microenvironment demonstrated that they had the ability to directly promote tumor progression and metastasis to the lung when present in tumor bearing hosts. The MISCs were shown to produce high levels of MMP9 and had the ability to integrate within the tumor vasculature. In addition, the incorporation into the tumor vasculature was further supported by induction of endothelial cell characteristics associated with MISCs in pro-angiogenic culture conditions which further demonstrated the plasticity of this immature myeloid derived cell population (Yang et al., 2004b). These results parallel independent observations associated with dendritic cells *in vivo* (Conejo-Garcia et al., 2004; Conejo-Garcia et al., 2005), or immature dendritic cells, monocytes and macrophages under pro-

angiogenic conditions *in vitro* (Fernandez Pujol et al., 2000; Fernandez Pujol et al., 2001; Schmeisser et al., 2001; Schmeisser and Strasser, 2002). The recently described roles for MISCs *in vivo*, supports and confirms previous observations that suggest host inflammatory responses significantly contribute to the production of angiogenic factors and proteinases that enhance tumor progression and metastasis (Coussens and Werb, 2002; Ono et al., 1999; Torisu et al., 2000). Importantly, it has been shown in a recent study conducted in our laboratory, that loss of the TGF-β response in mammary carcinoma cells can significantly enhance Cxcr2 dependent recruitment of tumor promoting MISCs to the mammary tumor microenvironment (Yang et al., 2008).

In addition to contributions associated with progression in the primary tumor microenvironment, myeloid derived cells in tumor bearing hosts have been reported to be associated with establishing a pre-metastatic niche in the lung that subsequently promotes tumor metastasis (Hiratsuka et al., 2002; Kaplan et al., 2006; Kaplan et al., 2005). Recently, it has been shown that primary tumors can produce circulating factors including TNF-α, VEGF-A and TGF-β1 that upregulate S100A8 and S100A9 production in the lung (Hiratsuka et al., 2006). S100A8 and S100A9 are known to be potent chemo-attractants and it has been shown that these factors promote recruitment of $Mac1^{+}$ cells to the pre-metastatic lung and promote tumor cell colonization of the lung tissue *in vivo* (Hiratsuka et al., 2006). This study, together with the previous results suggest that when bone marrow derived cells are present, they are able to significantly promote tumor progression and metastasis. Therefore, this diverse cell population should be further investigated and potentially targeted as an adjuvant therapeutic strategy to improve clinical prognoses and overall long-term survival.

## Summary and concluding remarks

We primarily examined the role for TGF-β signaling in MMTV-PyVmT induced mammary tumor initiation and progression through carcinoma progenitor cell specific deletion of TβRII *in vivo*. Using this strategy we have been able to show that when TGF-β signaling is lost in the mammary tumor microenvironment, several factors should be considered including the impact on carcinoma cell apoptosis, regulation of adjacent stromal fibrovascular cell populations, carcinoma cell lineage selection, regulation of inflammatory gene expression, and infiltration of tumor promoting bone marrow derived cell populations to the tumor microenvironment. It is likely that together, these compound factors significantly contribute to the TGF-β mediated regulation of early tumor progression and metastasis (Figure 30).

In systems designed to model human disease it is important to determine if the results can be linked to human disease. Therefore we designed a strategy that would allow us to correlate the loss of TGF-β signaling in carcinoma cells with the progression of human breast cancer. Toward this end, we have been able to determine that the global changes in gene expression when TGF-β signaling is lost in carcinoma cells likely results in a network of intrinsic, stromal-epithelial and host-tumor interactions that significantly regulate tumor progression and metastasis. In support of the importance for identifying global network interactions during tumor progression, our TGF-β signaling deficient gene expression signature significantly correlated with an increased risk of relapse in a breast cancer patients that exhibited Luminal A, ER+ or lymph node positive tumors at the time of tissue collection.

**Figure 30. Gain or loss of carcinoma cell specific TGF-β can promote tumor progression and metastasis.** TGF-β (β) can induce an epithelial to mesenchymal transition (EMT) that promotes metastasis. Loss of carcinoma cell specific TGF-β signaling (X) also increases metastasis and correlates with increased carcinoma cell apoptosis, regulation of adjacent stromal fibrovascular cell populations, carcinoma cell lineage selection, increased inflammatory gene expression and infiltration of tumor promoting bone marrow derived myeloid cell (BMDC) populations. In addition, the gene expression profile associated with the loss of carcinoma cell specific TGF-β signaling significantly correlated with reduced relapse-free survival in human breast cancer. Importantly, it is known that when human breast cancers do relapse they are often more aggressive and metastatic than the associated primary lesions. Together, our current data and those presented in the previous literature, demonstrate that gain or loss of TGF-β signaling in the mammary carcinoma cell population can promote metastasis.

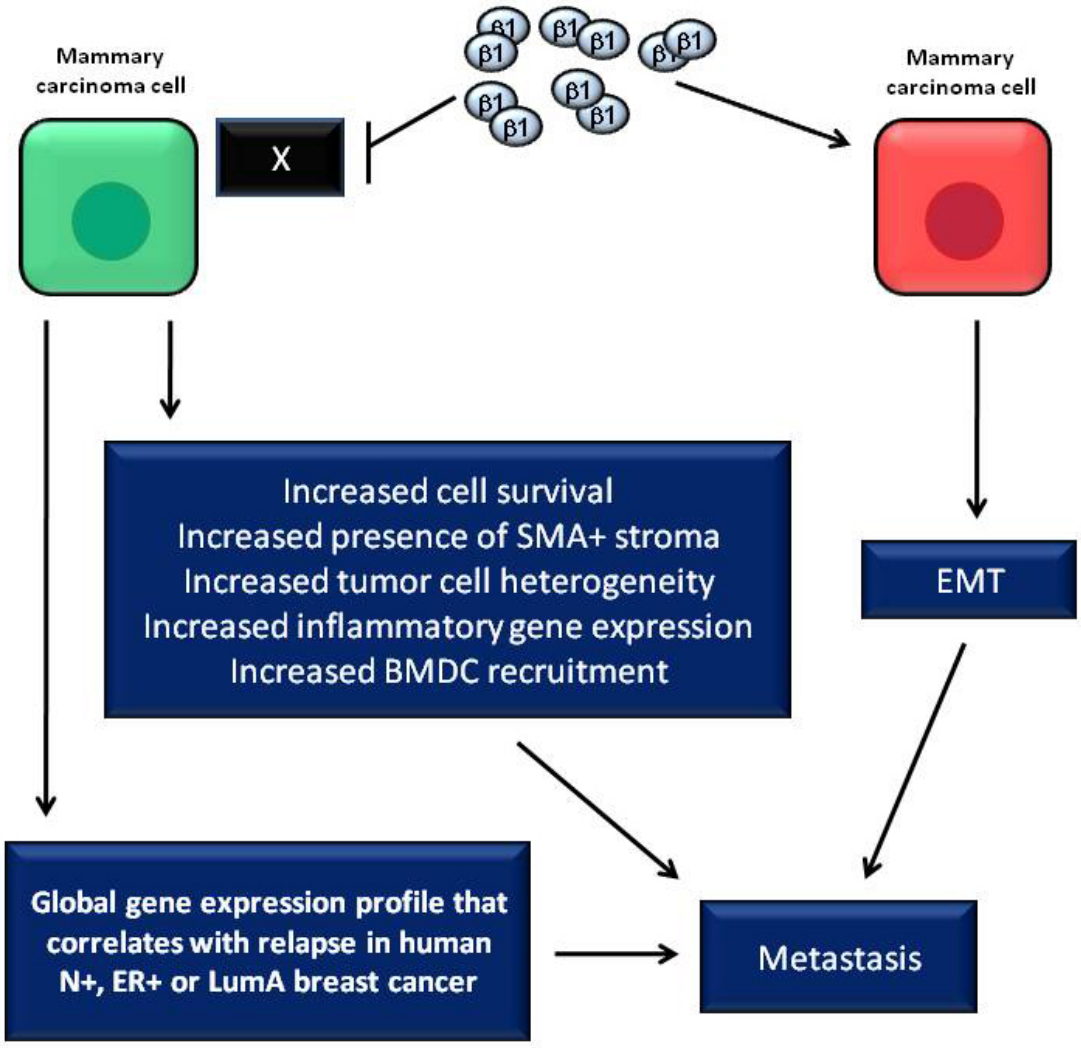


**Figure 30. Gain or loss of carcinoma cell specific TGF-β can promote tumor progression and metastasis.**

Our results suggested that a major contribution to the regulation of mammary tumor initiation and progression by TGF-β in our model system was stimulation of apoptosis (programmed cell death, type I). This is important, since TGF-β in normal epithelium is known to induce arrest of the cell cycle in G1, and during early tumor progression it has been suggested that this cytostatic regulation is a major contribution to carcinoma cell autonomous TGF-β mediated tumor suppression (Siegel and Massague, 2003). However, our data now suggests that the apoptotic response to TGF-β signaling also plays a significant role in early tumor suppression. In order to understand how the apoptotic gene regulation by TGF-β can intersect within the carcinoma cell it is necessary to have an understanding of Caspase dependent and independent mechanisms for induction of this process. Many of the mechanisms regulating Caspase dependent induction of apoptosis have been thoroughly reviewed (Boatright and Salvesen, 2003; Riedl and Salvesen, 2007; Schuster and Krieglstein, 2002). Briefly, Caspase 2, 8, 9 and 10 are initiator caspases while Caspases 3, 6 and 7 are considered to be executioner caspases. Caspases 8 and 10 are often associated with the extrinsic pathway while Caspases 2 and 9 are predominantly associated with the intrinsic pathway. Caspases 3 and 7 have been well studied whereas caspase 6 has not been characterized in as much detail. The extrinsic pathway is often initiated by death receptor activation and assembly of the death-inducing signaling complex (DISC) assembly. Assembly of the DISC complex promotes activation of caspase 8 and 10. The intrinsic pathway is activated by oxidative stress, ionizing radiation, some chemotherapies and mitochondrial damage. Activation of the intrinsic pathway is associated with mitochondrial membrane permeability that permits the release of cytochrome C and subsequent assembly of the

apoptosome. The apoptosome is a propeller like complex that has cytochrome C at the core bound to APAF-1 and caspase 9. Caspase 9 bound in the apoptosome cleaves and activates other caspase 9 proteins bound to the complex in trans. The activation of extrinsic or intrinsic Caspase pathways can lead to the activation of executioner caspases followed by cell death. In our tumor tissues however, there was no evidence of differential Caspase activation (data not shown). This supported the results in Chapter III demonstrating a predominant Caspase independent atypical PARP-1 cleavage product associated with the TβRII$^{(fl/fl);PY}$ tissues when compared with those in the TβRII$^{(WKO);PY}$ model. The atypical PARP-1 cleavage strongly suggested that μ-calpain may be more highly active, thereby mediating Caspase independent cell death, in TβRII$^{(fl/fl);PY}$ model when compared to those in the TβRII$^{(WKO);PY}$ model (Bentle et al., 2006) and Chapter III. Together, these results suggested that TGF-β can mediate mammary carcinoma cell death primarily through Caspase independent signaling.

In addition to μ-calpain activation, autophagy is a process present within carcinoma cells that can lead to programmed cell death (type II), and the mechanisms regulating this process have also been linked to Caspase independent signaling (Gozuacik and Kimchi, 2004). Prior to induction of cell death, it is currently thought that autophagy is a process wherein cells have the ability to digest their own organelles in nutrient deprived conditions to produce materials necessary for survival (Kondo and Kondo, 2006). Notably, we have now been able to demonstrate that the death associated protein kinase 2 (Dapk2; also known as DRP-1) is consistently upregulated in control TβRII$^{(fl/fl;PY)}$ tumor tissues when compared with those in the TβRII$^{(WKO;PY)}$ model (Chapter III). This was an important result, because Dapk2 is a calcium/calmodulin

dependent protein kinase that has been shown to induce autophagosome production and cell death independent of Caspase activation (Inbal et al., 2002; Inbal et al., 2000; Kondo and Kondo, 2006). Further, Dapk2 has been shown to promote cell death in response to IFN-γ, TNF-α, Fasl and TGF-β that are known to be produced by several cell populations within the tumor microenvironment (Gozuacik and Kimchi, 2004; Inbal et al., 2002) and Chapter I.

Together, our results presented in Chapter III suggested that TGF-β can regulate apoptosis and autophagy in mammary tumor tissues. This positively correlated with previous results produced in bovine mammary epithelial cells demonstrating that TGF-β1 stimulation could promote both apoptotic and autophagy associated cell death programs (Gajewska et al., 2005). Importantly, the regulation of cell death through either mechanism is clinically relevant since many conventional frontline chemotherapeutic agents are known to activate these pathways in the carcinoma cell population (Kondo and Kondo, 2006). Therefore, the resistance to programmed cell death may contribute to therapeutic resistance and recurrence when carcinoma cell response to TGF-β is lost *in vivo*. Further, resistance to programmed cell death may also permit the cells to travel better through the harsh environments outside of the primary tumor and consequently enable them to survive long enough to setup distant metastases.

In addition to intrinsic carcinoma cell signaling, the stromal microenvironment has recently been the focus of growing interest with regard to the regulation of tumor initiation, progression and metastasis. In the absence of carcinoma cell specific TGF-β signaling we were able to identify a significant difference in the stromal phenotype associated with adjacent stromal fibrovasculature. Further, we have now identified an

increase in the abundance of a F4/80+ bone marrow derived cell population in the tumors with carcinoma cell specific deletion of TβRII (Bierie et al., 2008; Yang et al., 2008) and Chapter III. We have been able to correlate the infiltration with increased expression of inflammatory genes including *Cxcl1*, *Cxcl5* and *Ptgs2* (COX-2) in the tumor tissues where TGF-β signaling has been deleted. Our current results suggest that regulation of inflammation is one of the major roles for TGF-β during tumor progression. Importantly, the F4/80 antigens have been widely used for the identification of macrophage lineage cell populations *in vivo*, and tumor-associated macrophages (TAMs) are known to significantly regulate normal mammary development and tumor progression (Condeelis and Pollard, 2006; Coussens and Werb, 2002; Lewis and Pollard, 2006).

Clinically, identification of TAM cell populations in invasive breast carcinoma tissue has been correlated with a poor prognosis that includes reduced relapse-free and overall survival (Leek et al., 1996). Monocytes can be recruited to the tumor microenvironment where they undergo limited macrophage differentiation and significantly contribute to tumor progression. It has been suggested that TAMs contribute to at least six central processes involved in tumor progression including tumor cell invasion, inflammation, matrix remodeling, intravasation, seeding at distant sites and promotion of angiogenesis (Condeelis and Pollard, 2006). Interestingly, it has been shown that carcinoma cells and tumor-associated macrophages have the ability to migrate together in response to the reciprocal expression of CSF-1 and EGF respectively (Wang et al., 2005a). Functionally, based on the literature it is clear that TAMs promote tumor progression and metastasis, therefore it is important to determine the factors produced by tumor cells that regulate the recruitment of TAMs to the tumor microenvironment. In

addition to previously described TAMs, recent work in our laboratory has now shown that TGF-β signaling in carcinoma cells can significantly regulate chemokine dependent recruitment of additional bone marrow derived cell lineages that contribute to tumor progression and metastasis (Yang et al., 2008).

In addition, we have identified a subtype of carcinoma cells with progenitor characteristics that were enriched in the mammary tumors that have a carcinoma progenitor cell specific deletion of TβRII *in vivo*. In the mammary gland, there is a common progenitor cell population that gives rise to ductal, lobular alveolar and myoepithelial cells. We have detected a population of cells in MMTV-PyVmT tumors that express K5 and dNp63, but lack the differentiation markers SMA and K8 that would be indicative of differentiation to myoepithelium and luminal epithelium respectively. It is likely that the K5+ dNp63+ SMA- K8- carcinoma cell subtype can be further stratified and we are currently working on techniques to sort these cells using putative stem cell markers such as CD44+ CD24- and Sca-1 with primary tumor isolates. Strikingly, we were able to detect an abundant K5+ dNp63+ cell population in the moderate to well differentiated MMTV-PyVmT lung metastases. This was unexpected due to the lobular and luminal morphology of the lung metastases in this tumor model. The presence of a basaloid cell population surrounding the moderate to well differentiated lobules in the lung metastases suggests that a *bona fide* carcinoma progenitor cell gave rise to the observed metastases.

In human breast cancer, loss of carcinoma cell specific response to TGF-β signaling has been linked to poor patient prognosis. However, many of the mechanisms through which TGF-β regulates these processes remain unknown. In an effort to address

this issue, we have now identified gene expression signatures indicative of ablated TGF-β signaling or TGF-β pathway activation in mammary carcinoma cells. The results suggest that TGF-β signaling mediates intrinsic, stromal-epithelial and host-tumor interactions during breast cancer progression. To determine the impact of our results relative to human breast cancer, we correlated our gene expression signatures with 1646 human breast cancer gene profiles and associated clinical data. Importantly, the TGF-β signaling deficient gene expression signature correlated with a significant increase in the risk of relapse for patients that presented with Luminal A, ER+ or lymph node positive breast cancer at the time of tissue collection.

The results we have reported herein, together with those reported in previous literature suggest that modification of the TGF-β pathway has a significant impact on the prognosis of human breast cancer progression. However at present, we do not have enough information to determine if therapeutic strategies involving this pathway will be beneficial for patients. Likely, the effect will depend upon the specific carcinoma cell alterations and stage of tumor progression present in each patient at the time of administration. However, our results strongly suggest that loss of TGF-β signaling can predispose women to post-operative relapse if they present with Luminal A, ER+ or lymph node positive breast cancer at the time of tissue collection. Consequently, if a TGF-β signaling deficiency is detected in a human mammary breast cancer our data may suggest that the patient should be treated more aggressively to decrease their risk of relapse and mortality.

# REFERENCES


**Abd El-Rehim, D. M., Pinder, S. E., Paish, C. E., Bell, J., Blamey, R. W., Robertson, J. F., Nicholson, R. I. and Ellis, I. O.** (2004). Expression of luminal and basal cytokeratins in human breast carcinoma. *J Pathol* **203**, 661-71.

**Adams, D. H., Hathaway, M., Shaw, J., Burnett, D., Elias, E. and Strain, A. J.** (1991). Transforming growth factor-beta induces human T lymphocyte migration in vitro. *J Immunol* **147**, 609-12.

**Ahmed, F., Wyckoff, J., Lin, E. Y., Wang, W., Wang, Y., Hennighausen, L., Miyazaki, J., Jones, J., Pollard, J. W., Condeelis, J. S. et al.** (2002). GFP expression in the mammary gland for imaging of mammary tumor cells in transgenic mice. *Cancer Res* **62**, 7166-9.

**Akhurst, R. J. and Derynck, R.** (2001). TGF-beta signaling in cancer--a double-edged sword. *Trends Cell Biol* **11**, S44-51.

**Alleva, D. G., Walker, T. M. and Elgert, K. D.** (1995). Induction of macrophage suppressor activity by fibrosarcoma-derived transforming growth factor-beta 1: contrasting effects on resting and activated macrophages. *J Leukoc Biol* **57**, 919-28.

**Allinen, M., Beroukhim, R., Cai, L., Brennan, C., Lahti-Domenici, J., Huang, H., Porter, D., Hu, M., Chin, L., Richardson, A. et al.** (2004). Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell* **6**, 17-32.

**Almand, B., Clark, J. I., Nikitina, E., van Beynen, J., English, N. R., Knight, S. C., Carbone, D. P. and Gabrilovich, D. I.** (2001). Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol* **166**, 678-89.

**Amendt, C., Schirmacher, P., Weber, H. and Blessing, M.** (1998). Expression of a dominant negative type II TGF-beta receptor in mouse skin results in an increase in carcinoma incidence and an acceleration of carcinoma development. *Oncogene* **17**, 25-34.

**Andrechek, E. R., Hardy, W. R., Siegel, P. M., Rudnicki, M. A., Cardiff, R. D. and Muller, W. J.** (2000). Amplification of the neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis. *Proc Natl Acad Sci U S A* **97**, 3444-9.

**Arteaga, C. L., Carty-Dugger, T., Moses, H. L., Hurd, S. D. and Pietenpol, J. A.** (1993a). Transforming growth factor beta 1 can induce estrogen-independent tumorigenicity of human breast cancer cells in athymic mice. *Cell Growth Differ* **4**, 193-201.

**Arteaga, C. L., Dugger, T. C., Winnier, A. R. and Forbes, J. T.** (1993b). Evidence for a positive role of transforming growth factor-beta in human breast cancer cell tumorigenesis. *J Cell Biochem Suppl* **17G**, 187-93.

**Arteaga, C. L., Hurd, S. D., Winnier, A. R., Johnson, M. D., Fendly, B. M. and Forbes, J. T.** (1993c). Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. *J Clin Invest* **92**, 2569-76.

**Bacon, A. L., Farrington, S. M. and Dunlop, M. G.** (2001). Mutation frequency in coding and non-coding repeat sequences in mismatch repair deficient cells derived from normal human tissue. *Oncogene* **20**, 7464-71.

**Bailey, J. P., Nieport, K. M., Herbst, M. P., Srivastava, S., Serra, R. A. and Horseman, N. D.** (2004). Prolactin and transforming growth factor-beta signaling exert opposing effects on

mammary gland morphogenesis, involution, and the Akt-forkhead pathway. *Mol Endocrinol* **18**, 1171-84.
**Bakin, A. V., Rinehart, C., Tomlinson, A. K. and Arteaga, C. L.** (2002). p38 mitogen-activated protein kinase is required for TGFbeta-mediated fibroblastic transdifferentiation and cell migration. *J Cell Sci* **115**, 3193-206.
**Bakin, A. V., Tomlinson, A. K., Bhowmick, N. A., Moses, H. L. and Arteaga, C. L.** (2000). Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* **275**, 36803-10.
**Bandyopadhyay, A., Lopez-Casillas, F., Malik, S. N., Montiel, J. L., Mendoza, V., Yang, J. and Sun, L. Z.** (2002). Antitumor activity of a recombinant soluble betaglycan in human breast cancer xenograft. *Cancer Res* **62**, 4690-5.
**Bandyopadhyay, A., Zhu, Y., Cibull, M. L., Bao, L., Chen, C. and Sun, L.** (1999). A soluble transforming growth factor beta type III receptor suppresses tumorigenicity and metastasis of human breast cancer MDA-MB-231 cells. *Cancer Res* **59**, 5041-6.
**Barbareschi, M., Pecciarini, L., Cangi, M. G., Macri, E., Rizzo, A., Viale, G. and Doglioni, C.** (2001). p63, a p53 homologue, is a selective nuclear marker of myoepithelial cells of the human breast. *Am J Surg Pathol* **25**, 1054-60.
**Barcellos-Hoff, M. H. and Ravani, S. A.** (2000). Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. *Cancer Res* **60**, 1254-60.
**Barlow, J., Yandell, D., Weaver, D., Casey, T. and Plaut, K.** (2003). Higher stromal expression of transforming growth factor-beta type II receptors is associated with poorer prognosis breast tumors. *Breast Cancer Res Treat* **79**, 149-59.
**Barrios-Rodiles, M., Brown, K. R., Ozdamar, B., Bose, R., Liu, Z., Donovan, R. S., Shinjo, F., Liu, Y., Dembowy, J., Taylor, I. W. et al.** (2005). High-throughput mapping of a dynamic signaling network in mammalian cells. *Science* **307**, 1621-5.
**Benckert, C., Jonas, S., Cramer, T., Von Marschall, Z., Schafer, G., Peters, M., Wagner, K., Radke, C., Wiedenmann, B., Neuhaus, P. et al.** (2003). Transforming growth factor beta 1 stimulates vascular endothelial growth factor gene transcription in human cholangiocellular carcinoma cells. *Cancer Res* **63**, 1083-92.
**Bentle, M. S., Bey, E. A., Dong, Y., Reinicke, K. E. and Boothman, D. A.** (2006). New tricks for old drugs: the anticarcinogenic potential of DNA repair inhibitors. *J Mol Histol* **37**, 203-18.
**Bey, E. A., Bentle, M. S., Reinicke, K. E., Dong, Y., Yang, C. R., Girard, L., Minna, J. D., Bornmann, W. G., Gao, J. and Boothman, D. A.** (2007). An NQO1- and PARP-1-mediated cell death pathway induced in non-small-cell lung cancer cells by beta-lapachone. *Proc Natl Acad Sci U S A* **104**, 11832-7.
**Bhowmick, N. A., Chytil, A., Plieth, D., Gorska, A. E., Dumont, N., Shappell, S., Washington, M. K., Neilson, E. G. and Moses, H. L.** (2004a). TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* **303**, 848-51.
**Bhowmick, N. A., Ghiassi, M., Bakin, A., Aakre, M., Lundquist, C. A., Engel, M. E., Arteaga, C. L. and Moses, H. L.** (2001). Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* **12**, 27-36.
**Bhowmick, N. A., Neilson, E. G. and Moses, H. L.** (2004b). Stromal fibroblasts in cancer initiation and progression. *Nature* **432**, 332-7.
**Bierie, B. and Moses, H. L.** (2006a). TGF-beta and cancer. *Cytokine Growth Factor Rev* **17**, 29-40.
**Bierie, B. and Moses, H. L.** (2006b). Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. *Nat Rev Cancer* **6**, 506-20.
**Bierie, B., Stover, D. G., Abel, T. W., Chytil, A., Gorska, A. E., Aakre, M., Forrester, E., Yang, L., Wagner, K. U. and Moses, H. L.** (2008). Transforming growth factor-beta regulates mammary

carcinoma cell survival and interaction with the adjacent microenvironment. *Cancer Res* **68**, 1809-19.
**Blobe, G. C., Schiemann, W. P. and Lodish, H. F.** (2000). Role of transforming growth factor beta in human disease. *N Engl J Med* **342**, 1350-8.
**Boatright, K. M. and Salvesen, G. S.** (2003). Mechanisms of caspase activation. *Curr Opin Cell Biol* **15**, 725-31.
**Bogdan, C. and Nathan, C.** (1993). Modulation of macrophage function by transforming growth factor beta, interleukin-4, and interleukin-10. *Ann N Y Acad Sci* **685**, 713-39.
**Bombara, C. and Ignotz, R. A.** (1992). TGF-beta inhibits proliferation of and promotes differentiation of human promonocytic leukemia cells. *J Cell Physiol* **153**, 30-7.
**Bottinger, E. P., Jakubczak, J. L., Haines, D. C., Bagnall, K. and Wakefield, L. M.** (1997a). Transgenic mice overexpressing a dominant-negative mutant type II transforming growth factor beta receptor show enhanced tumorigenesis in the mammary gland and lung in response to the carcinogen 7,12-dimethylbenz-[a]-anthracene. *Cancer Res* **57**, 5564-70.
**Bottinger, E. P., Jakubczak, J. L., Roberts, I. S., Mumy, M., Hemmati, P., Bagnall, K., Merlino, G. and Wakefield, L. M.** (1997b). Expression of a dominant-negative mutant TGF-beta type II receptor in transgenic mice reveals essential roles for TGF-beta in regulation of growth and differentiation in the exocrine pancreas. *Embo J* **16**, 2621-33.
**Boulanger, C. A. and Smith, G. H.** (2001). Reducing mammary cancer risk through premature stem cell senescence. *Oncogene* **20**, 2264-72.
**Boulanger, C. A., Wagner, K. U. and Smith, G. H.** (2005). Parity-induced mouse mammary epithelial cells are pluripotent, self-renewing and sensitive to TGF-beta1 expression. *Oncogene* **24**, 552-60.
**Breier, G., Blum, S., Peli, J., Groot, M., Wild, C., Risau, W. and Reichmann, E.** (2002). Transforming growth factor-beta and Ras regulate the VEGF/VEGF-receptor system during tumor angiogenesis. *Int J Cancer* **97**, 142-8.
**Brogi, E., Wu, T., Namiki, A. and Isner, J. M.** (1994). Indirect angiogenic cytokines upregulate VEGF and bFGF gene expression in vascular smooth muscle cells, whereas hypoxia upregulates VEGF expression only. *Circulation* **90**, 649-52.
**Bronte, V., Apolloni, E., Cabrelle, A., Ronca, R., Serafini, P., Zamboni, P., Restifo, N. P. and Zanovello, P.** (2000). Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells. *Blood* **96**, 3838-46.
**Brown, K. A., Aakre, M. E., Gorska, A. E., Price, J. O., Eltom, S. E., Pietenpol, J. A. and Moses, H. L.** (2004). Induction by transforming growth factor-beta1 of epithelial to mesenchymal transition is a rare event in vitro. *Breast Cancer Res* **6**, R215-31.
**Brown, S. L., Riehl, T. E., Walker, M. R., Geske, M. J., Doherty, J. M., Stenson, W. F. and Stappenbeck, T. S.** (2007). Myd88-dependent positioning of Ptgs2-expressing stromal cells maintains colonic epithelial proliferation during injury. *J Clin Invest* **117**, 258-69.
**Buckley, C. D., Amft, N., Bradfield, P. F., Pilling, D., Ross, E., Arenzana-Seisdedos, F., Amara, A., Curnow, S. J., Lord, J. M., Scheel-Toellner, D. et al.** (2000). Persistent induction of the chemokine receptor CXCR4 by TGF-beta 1 on synovial T cells contributes to their accumulation within the rheumatoid synovium. *J Immunol* **165**, 3423-9.
**Buono, K. D., Robinson, G. W., Martin, C., Shi, S., Stanley, P., Tanigaki, K., Honjo, T. and Hennighausen, L.** (2006). The canonical Notch/RBP-J signaling pathway controls the balance of cell lineages in mammary epithelium during pregnancy. *Dev Biol* **293**, 565-80.
**Calonge, M. J. and Massague, J.** (1999). Smad4/DPC4 silencing and hyperactive Ras jointly disrupt transforming growth factor-beta antiproliferative responses in colon cancer cells. *J Biol Chem* **274**, 33637-43.

**Candi, E., Dinsdale, D., Rufini, A., Salomoni, P., Knight, R. A., Mueller, M., Krammer, P. H. and Melino, G.** (2007). TAp63 and DeltaNp63 in cancer and epidermal development. *Cell Cycle* **6**, 274-85.
**Carroll, J. S., Meyer, C. A., Song, J., Li, W., Geistlinger, T. R., Eeckhoute, J., Brodsky, A. S., Keeton, E. K., Fertuck, K. C., Hall, G. F. et al.** (2006). Genome-wide analysis of estrogen receptor binding sites. *Nat Genet* **38**, 1289-97.
**Chambers, R. C., Leoni, P., Kaminski, N., Laurent, G. J. and Heller, R. A.** (2003). Global expression profiling of fibroblast responses to transforming growth factor-beta1 reveals the induction of inhibitor of differentiation-1 and provides evidence of smooth muscle cell phenotypic switching. *Am J Pathol* **162**, 533-46.
**Chang, H. Y., Chi, J. T., Dudoit, S., Bondre, C., van de Rijn, M., Botstein, D. and Brown, P. O.** (2002). Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc Natl Acad Sci U S A* **99**, 12877-82.
**Chapman, R. S., Lourenco, P. C., Tonner, E., Flint, D. J., Selbert, S., Takeda, K., Akira, S., Clarke, A. R. and Watson, C. J.** (1999). Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. *Genes Dev* **13**, 2604-16.
**Chen, J. J., Sun, Y. and Nabel, G. J.** (1998). Regulation of the proinflammatory effects of Fas ligand (CD95L). *Science* **282**, 1714-7.
**Chen, M. L., Pittet, M. J., Gorelik, L., Flavell, R. A., Weissleder, R., von Boehmer, H. and Khazaie, K.** (2005). Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. *Proc Natl Acad Sci U S A* **102**, 419-24.
**Chen, W. and Wahl, S. M.** (2003). TGF-beta: the missing link in CD4+CD25+ regulatory T cell-mediated immunosuppression. *Cytokine Growth Factor Rev* **14**, 85-9.
**Cheng, N., Bhowmick, N. A., Chytil, A., Gorksa, A. E., Brown, K. A., Muraoka, R., Arteaga, C. L., Neilson, E. G., Hayward, S. W. and Moses, H. L.** (2005). Loss of TGF-beta type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-alpha-, MSP- and HGF-mediated signaling networks. *Oncogene* **24**, 5053-68.
**Cheng, N., Chytil, A., Shyr, Y., Joly, A. and Moses, H. L.** (2007). Enhanced Hepatocyte Growth Factor Signaling by Type II Transforming Growth Factor-{beta} Receptor Knockout Fibroblasts Promotes Mammary Tumorigenesis. *Cancer Res* **67**, 4869-77.
**Chiu, C., Maddock, D. A., Zhang, Q., Souza, K. P., Townsend, A. R. and Wan, Y.** (2001). TGF-beta-induced p38 activation is mediated by Rac1-regulated generation of reactive oxygen species in cultured human keratinocytes. *Int J Mol Med* **8**, 251-5.
**Chomczynski, P. and Sacchi, N.** (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**, 156-9.
**Chytil, A., Magnuson, M. A., Wright, C. V. and Moses, H. L.** (2002). Conditional inactivation of the TGF-beta type II receptor using Cre:Lox. *Genesis* **32**, 73-5.
**Clarkson, R. W., Wayland, M. T., Lee, J., Freeman, T. and Watson, C. J.** (2004). Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. *Breast Cancer Res* **6**, R92-109.
**Cocolakis, E., Dai, M., Drevet, L., Ho, J., Haines, E., Ali, S. and Lebrun, J. J.** (2008). Smad signaling antagonizes STAT5-mediated gene transcription and mammary epithelial cell differentiation. *J Biol Chem* **283**, 1293-307.
**Condeelis, J. and Pollard, J. W.** (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124**, 263-6.
**Conejo-Garcia, J. R., Benencia, F., Courreges, M. C., Kang, E., Mohamed-Hadley, A., Buckanovich, R. J., Holtz, D. O., Jenkins, A., Na, H., Zhang, L. et al.** (2004). Tumor-infiltrating

dendritic cell precursors recruited by a beta-defensin contribute to vasculogenesis under the influence of Vegf-A. *Nat Med* **10**, 950-8.
**Conejo-Garcia, J. R., Buckanovich, R. J., Benencia, F., Courreges, M. C., Rubin, S. C., Carroll, R. G. and Coukos, G.** (2005). Vascular leukocytes contribute to tumor vascularization. *Blood* **105**, 679-81.
**Cordeiro, M. F., Gay, J. A. and Khaw, P. T.** (1999). Human anti-transforming growth factor-beta2 antibody: a new glaucoma anti-scarring agent. *Invest Ophthalmol Vis Sci* **40**, 2225-34.
**Cordenonsi, M., Dupont, S., Maretto, S., Insinga, A., Imbriano, C. and Piccolo, S.** (2003). Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads. *Cell* **113**, 301-14.
**Cordenonsi, M., Montagner, M., Adorno, M., Zacchigna, L., Martello, G., Mamidi, A., Soligo, S., Dupont, S. and Piccolo, S.** (2007). Integration of TGF-beta and Ras/MAPK signaling through p53 phosphorylation. *Science* **315**, 840-3.
**Cosgrove, D., Rodgers, K., Meehan, D., Miller, C., Bovard, K., Gilroy, A., Gardner, H., Kotelianski, V., Gotwals, P., Amatucci, A. et al.** (2000). Integrin alpha1beta1 and transforming growth factor-beta1 play distinct roles in alport glomerular pathogenesis and serve as dual targets for metabolic therapy. *Am J Pathol* **157**, 1649-59.
**Coussens, L. M. and Werb, Z.** (2002). Inflammation and cancer. *Nature* **420**, 860-7.
**Crawford, S. E., Stellmach, V., Murphy-Ullrich, J. E., Ribeiro, S. M., Lawler, J., Hynes, R. O., Boivin, G. P. and Bouck, N.** (1998). Thrombospondin-1 is a major activator of TGF-beta1 in vivo. *Cell* **93**, 1159-70.
**Crichton, M. B., Nichols, J. E., Zhao, Y., Bulun, S. E. and Simpson, E. R.** (1996). Expression of transcripts of interleukin-6 and related cytokines by human breast tumors, breast cancer cells, and adipose stromal cells. *Mol Cell Endocrinol* **118**, 215-20.
**Cui, W., Fowlis, D. J., Bryson, S., Duffie, E., Ireland, H., Balmain, A. and Akhurst, R. J.** (1996). TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. *Cell* **86**, 531-42.
**Cui, W., Fowlis, D. J., Cousins, F. M., Duffie, E., Bryson, S., Balmain, A. and Akhurst, R. J.** (1995). Concerted action of TGF-beta 1 and its type II receptor in control of epidermal homeostasis in transgenic mice. *Genes Dev* **9**, 945-55.
**Cunha, G. R., Reese, B. A. and Sekkingstad, M.** (1980). Induction of nuclear androgen-binding sites in epithelium of the embryonic urinary bladder by mesenchyme of the urogenital sinus of embryonic mice. *Endocrinology* **107**, 1767-70.
**Cunha, G. R., Young, P., Hom, Y. K., Cooke, P. S., Taylor, J. A. and Lubahn, D. B.** (1997). Elucidation of a role for stromal steroid hormone receptors in mammary gland growth and development using tissue recombinants. *J Mammary Gland Biol Neoplasia* **2**, 393-402.
**D'Angeac, A. D., Dornand, J., Emonds-Alt, X., Jullien, P., Garcia-Sanz, J. A. and Erard, F.** (1991). Transforming growth factor type beta 1 (TGF-beta 1) down-regulates interleukin-2 production and up-regulates interleukin-2 receptor expression in a thymoma cell line. *J Cell Physiol* **147**, 460-9.
**DaCosta Byfield, S., Major, C., Laping, N. J. and Roberts, A. B.** (2004). SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* **65**, 744-52.
**Daniel, C. W., Silberstein, G. B., Van Horn, K., Strickland, P. and Robinson, S.** (1989). TGF-beta 1-induced inhibition of mouse mammary ductal growth: developmental specificity and characterization. *Dev Biol* **135**, 20-30.

**Dasch, J. R., Pace, D. R., Waegell, W., Inenaga, D. and Ellingsworth, L.** (1989). Monoclonal antibodies recognizing transforming growth factor-beta. Bioactivity neutralization and transforming growth factor beta 2 affinity purification. *J Immunol* **142**, 1536-41.
**de Caestecker, M. P., Hemmati, P., Larisch-Bloch, S., Ajmera, R., Roberts, A. B. and Lechleider, R. J.** (1997). Characterization of functional domains within Smad4/DPC4. *J Biol Chem* **272**, 13690-6.
**de Caestecker, M. P., Piek, E. and Roberts, A. B.** (2000). Role of transforming growth factor-beta signaling in cancer. *J Natl Cancer Inst* **92**, 1388-402.
**de Jong, J. S., van Diest, P. J., van der Valk, P. and Baak, J. P.** (1998a). Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. *J Pathol* **184**, 53-7.
**de Jong, J. S., van Diest, P. J., van der Valk, P. and Baak, J. P.** (1998b). Expression of growth factors, growth inhibiting factors, and their receptors in invasive breast cancer. I: An inventory in search of autocrine and paracrine loops. *J Pathol* **184**, 44-52.
**de Visser, K. E. and Kast, W. M.** (1999). Effects of TGF-beta on the immune system: implications for cancer immunotherapy. *Leukemia* **13**, 1188-99.
**De Wever, O. and Mareel, M.** (2003). Role of tissue stroma in cancer cell invasion. *J Pathol* **200**, 429-47.
**De Wever, O., Nguyen, Q. D., Van Hoorde, L., Bracke, M., Bruyneel, E., Gespach, C. and Mareel, M.** (2004a). Tenascin-C and SF/HGF produced by myofibroblasts in vitro provide convergent pro-invasive signals to human colon cancer cells through RhoA and Rac. *Faseb J* **18**, 1016-8.
**De Wever, O., Westbroek, W., Verloes, A., Bloemen, N., Bracke, M., Gespach, C., Bruyneel, E. and Mareel, M.** (2004b). Critical role of N-cadherin in myofibroblast invasion and migration in vitro stimulated by colon-cancer-cell-derived TGF-beta or wounding. *J Cell Sci* **117**, 4691-703.
**Deckers, M., van Dinther, M., Buijs, J., Que, I., Lowik, C., van der Pluijm, G. and Ten Dijke, P.** (2006). The Tumor Suppressor Smad4 Is Required for Transforming Growth Factor {beta}-Induced Epithelial to Mesenchymal Transition and Bone Metastasis of Breast Cancer Cells. *Cancer Res* **66**, 2202-2209.
**Delens, N., Torreele, E., Savelkool, H., De Baetselier, P. and Bouwens, L.** (1994). Tumor-derived transforming growth factor-beta 1 and interleukin-6 are chemotactic for lymphokine-activated killer cells. *Int J Cancer* **57**, 696-700.
**Derynck, R., Akhurst, R. J. and Balmain, A.** (2001). TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* **29**, 117-29.
**Derynck, R., Goeddel, D. V., Ullrich, A., Gutterman, J. U., Williams, R. D., Bringman, T. S. and Berger, W. H.** (1987). Synthesis of messenger RNAs for transforming growth factors alpha and beta and the epidermal growth factor receptor by human tumors. *Cancer Res* **47**, 707-12.
**Derynck, R. and Zhang, Y. E.** (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**, 577-84.
**Desgrosellier, J. S., Mundell, N. A., McDonnell, M. A., Moses, H. L. and Barnett, J. V.** (2005). Activin receptor-like kinase 2 and Smad6 regulate epithelial-mesenchymal transformation during cardiac valve formation. *Dev Biol* **280**, 201-10.
**Dickson, R. B., Kasid, A., Huff, K. K., Bates, S. E., Knabbe, C., Bronzert, D., Gelmann, E. P. and Lippman, M. E.** (1987). Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17 beta-estradiol or v-Ha-ras oncogene. *Proc Natl Acad Sci U S A* **84**, 837-41.
**Donovan, D., Harmey, J. H., Toomey, D., Osborne, D. H., Redmond, H. P. and Bouchier-Hayes, D. J.** (1997). TGF beta-1 regulation of VEGF production by breast cancer cells. *Ann Surg Oncol* **4**, 621-7.

**Dumont, N. and Arteaga, C. L.** (2000). Transforming growth factor-beta and breast cancer: Tumor promoting effects of transforming growth factor-beta. *Breast Cancer Res* **2**, 125-32.
**Dumont, N., Bakin, A. V. and Arteaga, C. L.** (2003). Autocrine transforming growth factor-beta signaling mediates Smad-independent motility in human cancer cells. *J Biol Chem* **278**, 3275-85.
**Dupont, S., Zacchigna, L., Adorno, M., Soligo, S., Volpin, D., Piccolo, S. and Cordenonsi, M.** (2004). Convergence of p53 and TGF-beta signaling networks. *Cancer Lett* **213**, 129-38.
**Edlund, S., Landstrom, M., Heldin, C. H. and Aspenstrom, P.** (2004). Smad7 is required for TGF-beta-induced activation of the small GTPase Cdc42. *J Cell Sci* **117**, 1835-47.
**Engle, S. J., Hoying, J. B., Boivin, G. P., Ormsby, I., Gartside, P. S. and Doetschman, T.** (1999). Transforming growth factor beta1 suppresses nonmetastatic colon cancer at an early stage of tumorigenesis. *Cancer Res* **59**, 3379-86.
**Eppert, K., Scherer, S. W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L. C., Bapat, B., Gallinger, S., Andrulis, I. L. et al.** (1996). MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* **86**, 543-52.
**Ewan, K. B., Oketch-Rabah, H. A., Ravani, S. A., Shyamala, G., Moses, H. L. and Barcellos-Hoff, M. H.** (2005). Proliferation of estrogen receptor-alpha-positive mammary epithelial cells is restrained by transforming growth factor-beta1 in adult mice. *Am J Pathol* **167**, 409-17.
**Ewan, K. B., Shyamala, G., Ravani, S. A., Tang, Y., Akhurst, R., Wakefield, L. and Barcellos-Hoff, M. H.** (2002). Latent transforming growth factor-beta activation in mammary gland: regulation by ovarian hormones affects ductal and alveolar proliferation. *Am J Pathol* **160**, 2081-93.
**Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y. and Henson, P. M.** (1998). Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* **101**, 890-8.
**Fakhrai, H., Dorigo, O., Shawler, D. L., Lin, H., Mercola, D., Black, K. L., Royston, I. and Sobol, R. E.** (1996). Eradication of established intracranial rat gliomas by transforming growth factor beta antisense gene therapy. *Proc Natl Acad Sci U S A* **93**, 2909-14.
**Falcone, D. J., McCaffrey, T. A., Haimovitz-Friedman, A. and Garcia, M.** (1993). Transforming growth factor-beta 1 stimulates macrophage urokinase expression and release of matrix-bound basic fibroblast growth factor. *J Cell Physiol* **155**, 595-605.
**Feinberg, M. W., Shimizu, K., Lebedeva, M., Haspel, R., Takayama, K., Chen, Z., Frederick, J. P., Wang, X. F., Simon, D. I., Libby, P. et al.** (2004). Essential role for Smad3 in regulating MCP-1 expression and vascular inflammation. *Circ Res* **94**, 601-8.
**Feng, X. H. and Derynck, R.** (1996). Ligand-independent activation of transforming growth factor (TGF) beta signaling pathways by heteromeric cytoplasmic domains of TGF-beta receptors. *J Biol Chem* **271**, 13123-9.
**Feng, X. H. and Derynck, R.** (2005). Specificity and versatility in tgf-beta signaling through Smads. *Annu Rev Cell Dev Biol* **21**, 659-93.
**Feng, Z., Marti, A., Jehn, B., Altermatt, H. J., Chicaiza, G. and Jaggi, R.** (1995). Glucocorticoid and progesterone inhibit involution and programmed cell death in the mouse mammary gland. *J Cell Biol* **131**, 1095-103.
**Fernandez Pujol, B., Lucibello, F. C., Gehling, U. M., Lindemann, K., Weidner, N., Zuzarte, M. L., Adamkiewicz, J., Elsasser, H. P., Muller, R. and Havemann, K.** (2000). Endothelial-like cells derived from human CD14 positive monocytes. *Differentiation* **65**, 287-300.
**Fernandez Pujol, B., Lucibello, F. C., Zuzarte, M., Lutjens, P., Muller, R. and Havemann, K.** (2001). Dendritic cells derived from peripheral monocytes express endothelial markers and in

the presence of angiogenic growth factors differentiate into endothelial-like cells. *Eur J Cell Biol* **80**, 99-110.
**Fontana, A., Bodmer, S., Frei, K., Malipiero, U. and Siepl, C.** (1991). Expression of TGF-beta 2 in human glioblastoma: a role in resistance to immune rejection? *Ciba Found Symp* **157**, 232-8; discussion 238-41.
**Fontana, A., Constam, D. B., Frei, K., Malipiero, U. and Pfister, H. W.** (1992). Modulation of the immune response by transforming growth factor beta. *Int Arch Allergy Immunol* **99**, 1-7.
**Forrester, E., Chytil, A., Bierie, B., Aakre, M., Gorska, A. E., Sharif-Afshar, A. R., Muller, W. J. and Moses, H. L.** (2005). Effect of conditional knockout of the type II TGF-beta receptor gene in mammary epithelia on mammary gland development and polyomavirus middle T antigen induced tumor formation and metastasis. *Cancer Res* **65**, 2296-302.
**Fowlis, D. J., Cui, W., Johnson, S. A., Balmain, A. and Akhurst, R. J.** (1996). Altered epidermal cell growth control in vivo by inducible expression of transforming growth factor beta 1 in the skin of transgenic mice. *Cell Growth Differ* **7**, 679-87.
**Fukino, K., Shen, L., Matsumoto, S., Morrison, C. D., Mutter, G. L. and Eng, C.** (2004). Combined total genome loss of heterozygosity scan of breast cancer stroma and epithelium reveals multiplicity of stromal targets. *Cancer Res* **64**, 7231-6.
**Fulda, S. and Debatin, K. M.** (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene* **25**, 4798-811.
**Gabrilovich, D. I., Chen, H. L., Girgis, K. R., Cunningham, H. T., Meny, G. M., Nadaf, S., Kavanaugh, D. and Carbone, D. P.** (1996). Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med* **2**, 1096-103.
**Gajewska, M., Gajkowska, B. and Motyl, T.** (2005). Apoptosis and autophagy induced by TGF-B1 in bovine mammary epithelial BME-UV1 cells. *J Physiol Pharmacol* **56 Suppl 3**, 143-57.
**Gallego, M. I., Bierie, B. and Hennighausen, L.** (2003). Targeted expression of HGF/SF in mouse mammary epithelium leads to metastatic adenosquamous carcinomas through the activation of multiple signal transduction pathways. *Oncogene* **22**, 8498-508.
**Ge, R., Rajeev, V., Subramanian, G., Reiss, K. A., Liu, D., Higgins, L., Joly, A., Dugar, S., Chakravarty, J., Henson, M. et al.** (2004). Selective inhibitors of type I receptor kinase block cellular transforming growth factor-beta signaling. *Biochem Pharmacol* **68**, 41-50.
**Geiser, A. G., Letterio, J. J., Kulkarni, A. B., Karlsson, S., Roberts, A. B. and Sporn, M. B.** (1993). Transforming growth factor beta 1 (TGF-beta 1) controls expression of major histocompatibility genes in the postnatal mouse: aberrant histocompatibility antigen expression in the pathogenesis of the TGF-beta 1 null mouse phenotype. *Proc Natl Acad Sci U S A* **90**, 9944-8.
**Gerdes, M. J., Larsen, M., Dang, T. D., Ressler, S. J., Tuxhorn, J. A. and Rowley, D. R.** (2004). Regulation of rat prostate stromal cell myodifferentiation by androgen and TGF-beta1. *Prostate* **58**, 299-307.
**Giannouli, C. C. and Kletsas, D.** (2005). TGF-beta regulates differentially the proliferation of fetal and adult human skin fibroblasts via the activation of PKA and the autocrine action of FGF-2. *Cell Signal*.
**Gilboa, L., Wells, R. G., Lodish, H. F. and Henis, Y. I.** (1998). Oligomeric structure of type I and type II transforming growth factor beta receptors: homodimers form in the ER and persist at the plasma membrane. *J Cell Biol* **140**, 767-77.
**Go, C., He, W., Zhong, L., Li, P., Huang, J., Brinkley, B. R. and Wang, X. J.** (2000). Aberrant cell cycle progression contributes to the early-stage accelerated carcinogenesis in transgenic epidermis expressing the dominant negative TGFbetaRII. *Oncogene* **19**, 3623-31.

**Go, C., Li, P. and Wang, X. J.** (1999). Blocking transforming growth factor beta signaling in transgenic epidermis accelerates chemical carcinogenesis: a mechanism associated with increased angiogenesis. *Cancer Res* **59**, 2861-8.
**Gobbi, H., Arteaga, C. L., Jensen, R. A., Simpson, J. F., Dupont, W. D., Olson, S. J., Schuyler, P. A., Plummer, W. D., Jr. and Page, D. L.** (2000). Loss of expression of transforming growth factor beta type II receptor correlates with high tumour grade in human breast in-situ and invasive carcinomas. *Histopathology* **36**, 168-77.
**Gobbi, H., Dupont, W. D., Simpson, J. F., Plummer, W. D., Jr., Schuyler, P. A., Olson, S. J., Arteaga, C. L. and Page, D. L.** (1999). Transforming growth factor-beta and breast cancer risk in women with mammary epithelial hyperplasia. *J Natl Cancer Inst* **91**, 2096-101.
**Gorelik, L. and Flavell, R. A.** (2000). Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* **12**, 171-81.
**Gorelik, L. and Flavell, R. A.** (2001). Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med* **7**, 1118-22.
**Gorelik, L. and Flavell, R. A.** (2002). Transforming growth factor-beta in T-cell biology. *Nat Rev Immunol* **2**, 46-53.
**Gorska, A. E., Jensen, R. A., Shyr, Y., Aakre, M. E., Bhowmick, N. A. and Moses, H. L.** (2003). Transgenic mice expressing a dominant-negative mutant type II transforming growth factor-beta receptor exhibit impaired mammary development and enhanced mammary tumor formation. *Am J Pathol* **163**, 1539-49.
**Gorska, A. E., Joseph, H., Derynck, R., Moses, H. L. and Serra, R.** (1998). Dominant-negative interference of the transforming growth factor beta type II receptor in mammary gland epithelium results in alveolar hyperplasia and differentiation in virgin mice. *Cell Growth Differ* **9**, 229-38.
**Goumans, M. J., Lebrin, F. and Valdimarsdottir, G.** (2003a). Controlling the angiogenic switch: a balance between two distinct TGF-b receptor signaling pathways. *Trends Cardiovasc Med* **13**, 301-7.
**Goumans, M. J., Valdimarsdottir, G., Itoh, S., Lebrin, F., Larsson, J., Mummery, C., Karlsson, S. and ten Dijke, P.** (2003b). Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. *Mol Cell* **12**, 817-28.
**Gozuacik, D. and Kimchi, A.** (2004). Autophagy as a cell death and tumor suppressor mechanism. *Oncogene* **23**, 2891-906.
**Grady, W. M.** (2004). Genomic instability and colon cancer. *Cancer Metastasis Rev* **23**, 11-27.
**Grady, W. M., Myeroff, L. L., Swinler, S. E., Rajput, A., Thiagalingam, S., Lutterbaugh, J. D., Neumann, A., Brattain, M. G., Chang, J., Kim, S. J. et al.** (1999). Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Res* **59**, 320-4.
**Gruber, B. L., Marchese, M. J. and Kew, R. R.** (1994). Transforming growth factor-beta 1 mediates mast cell chemotaxis. *J Immunol* **152**, 5860-7.
**Guy, C. T., Cardiff, R. D. and Muller, W. J.** (1992a). Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol Cell Biol* **12**, 954-61.
**Guy, C. T., Webster, M. A., Schaller, M., Parsons, T. J., Cardiff, R. D. and Muller, W. J.** (1992b). Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci U S A* **89**, 10578-82.
**Haak-Frendscho, M., Wynn, T. A., Czuprynski, C. J. and Paulnock, D.** (1990). Transforming growth factor-beta 1 inhibits activation of macrophage cell line RAW 264.7 for cell killing. *Clin Exp Immunol* **82**, 404-10.

**Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H. et al.** (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* **271**, 350-3.
**Hahne, M., Rimoldi, D., Schroter, M., Romero, P., Schreier, M., French, L. E., Schneider, P., Bornand, T., Fontana, A., Lienard, D. et al.** (1996). Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. *Science* **274**, 1363-6.
**Han, G., Lu, S. L., Li, A. G., He, W., Corless, C. L., Kulesz-Martin, M. and Wang, X. J.** (2005). Distinct mechanisms of TGF-beta1-mediated epithelial-to-mesenchymal transition and metastasis during skin carcinogenesis. *J Clin Invest* **115**, 1714-23.
**Han, S. U., Kim, H. T., Seong do, H., Kim, Y. S., Park, Y. S., Bang, Y. J., Yang, H. K. and Kim, S. J.** (2004). Loss of the Smad3 expression increases susceptibility to tumorigenicity in human gastric cancer. *Oncogene* **23**, 1333-41.
**Hanahan, D. and Folkman, J.** (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353-64.
**Hanahan, D. and Weinberg, R. A.** (2000). The hallmarks of cancer. *Cell* **100**, 57-70.
**Hanazawa, S., Takeshita, A., Tsukamoto, Y., Kawata, Y., Takara, K. O. and Kitano, S.** (1991). Transforming growth factor-beta-induced gene expression of monocyte chemoattractant JE in mouse osteoblastic cells, MC3T3-E1. *Biochem Biophys Res Commun* **180**, 1130-6.
**Hayward, S. W., Wang, Y., Cao, M., Hom, Y. K., Zhang, B., Grossfeld, G. D., Sudilovsky, D. and Cunha, G. R.** (2001). Malignant transformation in a nontumorigenic human prostatic epithelial cell line. *Cancer Res* **61**, 8135-42.
**Hennighausen, L. and Robinson, G. W.** (2005). Information networks in the mammary gland. *Nat Rev Mol Cell Biol* **6**, 715-25.
**Henry, M. D., Triplett, A. A., Oh, K. B., Smith, G. H. and Wagner, K. U.** (2004). Parity-induced mammary epithelial cells facilitate tumorigenesis in MMTV-neu transgenic mice. *Oncogene* **23**, 6980-5.
**Hildenbrand, R., Jansen, C., Wolf, G., Bohme, B., Berger, S., von Minckwitz, G., Horlin, A., Kaufmann, M. and Stutte, H. J.** (1998). Transforming growth factor-beta stimulates urokinase expression in tumor-associated macrophages of the breast. *Lab Invest* **78**, 59-71.
**Hill, R., Song, Y., Cardiff, R. D. and Van Dyke, T.** (2005). Selective evolution of stromal mesenchyme with p53 loss in response to epithelial tumorigenesis. *Cell* **123**, 1001-11.
**Hira, E., Ono, T., Dhar, D. K., El-Assal, O. N., Hishikawa, Y., Yamanoi, A. and Nagasue, N.** (2005). Overexpression of macrophage migration inhibitory factor induces angiogenesis and deteriorates prognosis after radical resection for hepatocellular carcinoma. *Cancer* **103**, 588-98.
**Hiratsuka, S., Nakamura, K., Iwai, S., Murakami, M., Itoh, T., Kijima, H., Shipley, J. M., Senior, R. M. and Shibuya, M.** (2002). MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. *Cancer Cell* **2**, 289-300.
**Hiratsuka, S., Watanabe, A., Aburatani, H. and Maru, Y.** (2006). Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. *Nat Cell Biol* **8**, 1369-75.
**Holzer, R. G., Ryan, R. E., Tommack, M., Schlekeway, E. and Jorcyk, C. L.** (2004). Oncostatin M stimulates the detachment of a reservoir of invasive mammary carcinoma cells: role of cyclooxygenase-2. *Clin Exp Metastasis* **21**, 167-76.
**Howe, J. R., Roth, S., Ringold, J. C., Summers, R. W., Jarvinen, H. J., Sistonen, P., Tomlinson, I. P., Houlston, R. S., Bevan, S., Mitros, F. A. et al.** (1998). Mutations in the SMAD4/DPC4 gene in juvenile polyposis. *Science* **280**, 1086-8.

**Howe, J. R., Shellnut, J., Wagner, B., Ringold, J. C., Sayed, M. G., Ahmed, A. F., Lynch, P. M., Amos, C. I., Sistonen, P. and Aaltonen, L. A.** (2002). Common deletion of SMAD4 in juvenile polyposis is a mutational hotspot. *Am J Hum Genet* **70**, 1357-62.
**Hsiao, Y. W., Liao, K. W., Hung, S. W. and Chu, R. M.** (2004). Tumor-infiltrating lymphocyte secretion of IL-6 antagonizes tumor-derived TGF-beta 1 and restores the lymphokine-activated killing activity. *J Immunol* **172**, 1508-14.
**Hu, M., Yao, J., Cai, L., Bachman, K. E., van den Brule, F., Velculescu, V. and Polyak, K.** (2005). Distinct epigenetic changes in the stromal cells of breast cancers. *Nat Genet* **37**, 899-905.
**Humphreys, R. C., Bierie, B., Zhao, L., Raz, R., Levy, D. and Hennighausen, L.** (2002). Deletion of Stat3 blocks mammary gland involution and extends functional competence of the secretory epithelium in the absence of lactogenic stimuli. *Endocrinology* **143**, 3641-50.
**Humphreys, R. C., Lydon, J., O'Malley, B. W. and Rosen, J. M.** (1997). Mammary gland development is mediated by both stromal and epithelial progesterone receptors. *Mol Endocrinol* **11**, 801-11.
**Inbal, B., Bialik, S., Sabanay, I., Shani, G. and Kimchi, A.** (2002). DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. *J Cell Biol* **157**, 455-68.
**Inbal, B., Shani, G., Cohen, O., Kissil, J. L. and Kimchi, A.** (2000). Death-associated protein kinase-related protein 1, a novel serine/threonine kinase involved in apoptosis. *Mol Cell Biol* **20**, 1044-54.
**Inman, G. J., Nicolas, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Laping, N. J. and Hill, C. S.** (2002). SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* **62**, 65-74.
**Ivshina, A. V., George, J., Senko, O., Mow, B., Putti, T. C., Smeds, J., Lindahl, T., Pawitan, Y., Hall, P., Nordgren, H. et al.** (2006). Genetic reclassification of histologic grade delineates new clinical subtypes of breast cancer. *Cancer Res* **66**, 10292-301.
**Jhappan, C., Geiser, A. G., Kordon, E. C., Bagheri, D., Hennighausen, L., Roberts, A. B., Smith, G. H. and Merlino, G.** (1993). Targeting expression of a transforming growth factor beta 1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. *Embo J* **12**, 1835-45.
**Johns, L. D., Babcock, G., Green, D., Freedman, M., Sriram, S. and Ransohoff, R. M.** (1992). Transforming growth factor-beta 1 differentially regulates proliferation and MHC class-II antigen expression in forebrain and brainstem astrocyte primary cultures. *Brain Res* **585**, 229-36.
**Jorcyk, C. L., Holzer, R. G. and Ryan, R. E.** (2006). Oncostatin M induces cell detachment and enhances the metastatic capacity of T-47D human breast carcinoma cells. *Cytokine* **33**, 323-36.
**Joseph, H., Gorska, A. E., Sohn, P., Moses, H. L. and Serra, R.** (1999). Overexpression of a kinase-deficient transforming growth factor-beta type II receptor in mouse mammary stroma results in increased epithelial branching. *Mol Biol Cell* **10**, 1221-34.
**Kang, S. H., Bang, Y. J., Im, Y. H., Yang, H. K., Lee, D. A., Lee, H. Y., Lee, H. S., Kim, N. K. and Kim, S. J.** (1999). Transcriptional repression of the transforming growth factor-beta type I receptor gene by DNA methylation results in the development of TGF-beta resistance in human gastric cancer. *Oncogene* **18**, 7280-6.
**Kaplan, R. N., Psaila, B. and Lyden, D.** (2006). Bone marrow cells in the 'pre-metastatic niche': within bone and beyond. *Cancer Metastasis Rev* **25**, 521-9.
**Kaplan, R. N., Riba, R. D., Zacharoulis, S., Bramley, A. H., Vincent, L., Costa, C., MacDonald, D. D., Jin, D. K., Shido, K., Kerns, S. A. et al.** (2005). VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* **438**, 820-7.

**Kawanishi, H., Matsui, Y., Ito, M., Watanabe, J., Takahashi, T., Nishizawa, K., Nishiyama, H., Kamoto, T., Mikami, Y., Tanaka, Y. et al.** (2008). Secreted CXCL1 is a potential mediator and marker of the tumor invasion of bladder cancer. *Clin Cancer Res* **14**, 2579-87.
**Kim, B. G., Li, C., Qiao, W., Mamura, M., Kasperczak, B., Anver, M., Wolfraim, L., Hong, S., Mushinski, E., Potter, M. et al.** (2006). Smad4 signalling in T cells is required for suppression of gastrointestinal cancer. *Nature* **441**, 1015-9.
**Kim, S. J., Im, Y. H., Markowitz, S. D. and Bang, Y. J.** (2000). Molecular mechanisms of inactivation of TGF-beta receptors during carcinogenesis. *Cytokine Growth Factor Rev* **11**, 159-68.
**Kitamura, M.** (1997). Identification of an inhibitor targeting macrophage production of monocyte chemoattractant protein-1 as TGF-beta 1. *J Immunol* **159**, 1404-11.
**Kitamura, S., Maeshima, Y., Sugaya, T., Sugiyama, H., Yamasaki, Y. and Makino, H.** (2003). Transforming growth factor-beta 1 induces vascular endothelial growth factor expression in murine proximal tubular epithelial cells. *Nephron Exp Nephrol* **95**, e79-86.
**Kolek, O., Gajkowska, B., Godlewski, M. M. and Tomasz, M.** (2003). Co-localization of apoptosis-regulating proteins in mouse mammary epithelial HC11 cells exposed to TGF-beta1. *Eur J Cell Biol* **82**, 303-12.
**Kondo, Y. and Kondo, S.** (2006). Autophagy and cancer therapy. *Autophagy* **2**, 85-90.
**Kordon, E. C., McKnight, R. A., Jhappan, C., Hennighausen, L., Merlino, G. and Smith, G. H.** (1995). Ectopic TGF beta 1 expression in the secretory mammary epithelium induces early senescence of the epithelial stem cell population. *Dev Biol* **168**, 47-61.
**Kretzschmar, M.** (2000). Transforming growth factor-beta and breast cancer: Transforming growth factor-beta/SMAD signaling defects and cancer. *Breast Cancer Res* **2**, 107-15.
**Kulkarni, A. B., Huh, C. G., Becker, D., Geiser, A., Lyght, M., Flanders, K. C., Roberts, A. B., Sporn, M. B., Ward, J. M. and Karlsson, S.** (1993). Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci U S A* **90**, 770-4.
**Kuperwasser, C., Chavarria, T., Wu, M., Magrane, G., Gray, J. W., Carey, L., Richardson, A. and Weinberg, R. A.** (2004). Reconstruction of functionally normal and malignant human breast tissues in mice. *Proc Natl Acad Sci U S A* **101**, 4966-71.
**Kusmartsev, S. and Gabrilovich, D. I.** (2002). Immature myeloid cells and cancer-associated immune suppression. *Cancer Immunol Immunother* **51**, 293-8.
**Kusmartsev, S. and Gabrilovich, D. I.** (2003). Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species. *J Leukoc Biol* **74**, 186-96.
**Lafontant, P. J., Burns, A. R., Donnachie, E., Haudek, S. B., Smith, C. W. and Entman, M. L.** (2006). Oncostatin M differentially regulates CXC chemokines in mouse cardiac fibroblasts. *Am J Physiol Cell Physiol* **291**, C18-26.
**Lai, Y. T., Beason, K. B., Brames, G. P., Desgrosellier, J. S., Cleggett, M. C., Shaw, M. V., Brown, C. B. and Barnett, J. V.** (2000). Activin receptor-like kinase 2 can mediate atrioventricular cushion transformation. *Dev Biol* **222**, 1-11.
**Landis, M. D., Seachrist, D. D., Montanez-Wiscovich, M. E., Danielpour, D. and Keri, R. A.** (2005). Gene expression profiling of cancer progression reveals intrinsic regulation of transforming growth factor-beta signaling in ErbB2/Neu-induced tumors from transgenic mice. *Oncogene* **24**, 5173-90.
**Lean, J. M., Murphy, C., Fuller, K. and Chambers, T. J.** (2002). CCL9/MIP-1gamma and its receptor CCR1 are the major chemokine ligand/receptor species expressed by osteoclasts. *J Cell Biochem* **87**, 386-93.

**Lebrin, F., Deckers, M., Bertolino, P. and Ten Dijke, P.** (2005). TGF-beta receptor function in the endothelium. *Cardiovasc Res* **65**, 599-608.
**Lee, Y. J., Han, Y., Lu, H. T., Nguyen, V., Qin, H., Howe, P. H., Hocevar, B. A., Boss, J. M., Ransohoff, R. M. and Benveniste, E. N.** (1997). TGF-beta suppresses IFN-gamma induction of class II MHC gene expression by inhibiting class II transactivator messenger RNA expression. *J Immunol* **158**, 2065-75.
**Leek, R. D., Lewis, C. E., Whitehouse, R., Greenall, M., Clarke, J. and Harris, A. L.** (1996). Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res* **56**, 4625-9.
**Lenferink, A. E., Magoon, J., Pepin, M. C., Guimond, A. and O'Connor-McCourt, M. D.** (2003). Expression of TGF-beta type II receptor antisense RNA impairs TGF-beta signaling in vitro and promotes mammary gland differentiation in vivo. *Int J Cancer* **107**, 919-28.
**Leof, E. B., Proper, J. A., Goustin, A. S., Shipley, G. D., DiCorleto, P. E. and Moses, H. L.** (1986). Induction of c-sis mRNA and activity similar to platelet-derived growth factor by transforming growth factor beta: a proposed model for indirect mitogenesis involving autocrine activity. *Proc Natl Acad Sci U S A* **83**, 2453-7.
**Leveen, P., Larsson, J., Ehinger, M., Cilio, C. M., Sundler, M., Sjostrand, L. J., Holmdahl, R. and Karlsson, S.** (2002). Induced disruption of the transforming growth factor beta type II receptor gene in mice causes a lethal inflammatory disorder that is transplantable. *Blood* **100**, 560-8.
**Levy, L. and Hill, C. S.** (2006). Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. *Cytokine Growth Factor Rev* **17**, 41-58.
**Lewis, C. E. and Pollard, J. W.** (2006). Distinct role of macrophages in different tumor microenvironments. *Cancer Res* **66**, 605-12.
**Lewis, M. P., Lygoe, K. A., Nystrom, M. L., Anderson, W. P., Speight, P. M., Marshall, J. F. and Thomas, G. J.** (2004). Tumour-derived TGF-beta1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. *Br J Cancer* **90**, 822-32.
**Li, A., Varney, M. L. and Singh, R. K.** (2004). Constitutive expression of growth regulated oncogene (gro) in human colon carcinoma cells with different metastatic potential and its role in regulating their metastatic phenotype. *Clin Exp Metastasis* **21**, 571-9.
**Li, W., Qiao, W., Chen, L., Xu, X., Yang, X., Li, D., Li, C., Brodie, S. G., Meguid, M. M., Hennighausen, L. et al.** (2003). Squamous cell carcinoma and mammary abscess formation through squamous metaplasia in Smad4/Dpc4 conditional knockout mice. *Development* **130**, 6143-53.
**Liau, L. M., Fakhrai, H. and Black, K. L.** (1998). Prolonged survival of rats with intracranial C6 gliomas by treatment with TGF-beta antisense gene. *Neurol Res* **20**, 742-7.
**Lin, H. K., Bergmann, S. and Pandolfi, P. P.** (2004). Cytoplasmic PML function in TGF-beta signalling. *Nature* **431**, 205-11.
**Lin, H. Y., Moustakas, A., Knaus, P., Wells, R. G., Henis, Y. I. and Lodish, H. F.** (1995). The soluble exoplasmic domain of the type II transforming growth factor (TGF)-beta receptor. A heterogeneously glycosylated protein with high affinity and selectivity for TGF-beta ligands. *J Biol Chem* **270**, 2747-54.
**Lin, H. Y., Wang, X. F., Ng-Eaton, E., Weinberg, R. A. and Lodish, H. F.** (1992). Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase. *Cell* **68**, 775-85.
**Loi, S., Haibe-Kains, B., Desmedt, C., Lallemand, F., Tutt, A. M., Gillet, C., Ellis, P., Harris, A., Bergh, J., Foekens, J. A. et al.** (2007). Definition of clinically distinct molecular subtypes in estrogen receptor-positive breast carcinomas through genomic grade. *J Clin Oncol* **25**, 1239-46.

**Loi, S., Haibe-Kains, B., Desmedt, C., Wirapati, P., Lallemand, F., Tutt, A. M., Gillet, C., Ellis, P., Ryder, K., Reid, J. F. et al.** (2008). Predicting prognosis using molecular profiling in estrogen receptor-positive breast cancer treated with tamoxifen. *BMC Genomics* **9**, 239.
**Long, W., Wagner, K. U., Lloyd, K. C., Binart, N., Shillingford, J. M., Hennighausen, L. and Jones, F. E.** (2003). Impaired differentiation and lactational failure of Erbb4-deficient mammary glands identify ERBB4 as an obligate mediator of STAT5. *Development* **130**, 5257-68.
**Lopez, M. J. and Porter, K. A.** (1996). Inflammatory breast cancer. *Surg Clin North Am* **76**, 411-29.
**Lucas, C., Bald, L. N., Fendly, B. M., Mora-Worms, M., Figari, I. S., Patzer, E. J. and Palladino, M. A.** (1990). The autocrine production of transforming growth factor-beta 1 during lymphocyte activation. A study with a monoclonal antibody-based ELISA. *J Immunol* **145**, 1415-22.
**Lucke, C. D., Philpott, A., Metcalfe, J. C., Thompson, A. M., Hughes-Davies, L., Kemp, P. R. and Hesketh, R.** (2001). Inhibiting mutations in the transforming growth factor beta type 2 receptor in recurrent human breast cancer. *Cancer Res* **61**, 482-5.
**Luo, K. and Lodish, H. F.** (1996). Signaling by chimeric erythropoietin-TGF-beta receptors: homodimerization of the cytoplasmic domain of the type I TGF-beta receptor and heterodimerization with the type II receptor are both required for intracellular signal transduction. *Embo J* **15**, 4485-96.
**Lygoe, K. A., Norman, J. T., Marshall, J. F. and Lewis, M. P.** (2004). AlphaV integrins play an important role in myofibroblast differentiation. *Wound Repair Regen* **12**, 461-70.
**Ma, D. and Niederkorn, J. Y.** (1995). Transforming growth factor-beta down-regulates major histocompatibility complex class I antigen expression and increases the susceptibility of uveal melanoma cells to natural killer cell-mediated cytolysis. *Immunology* **86**, 263-9.
**MacGrogan, D., Pegram, M., Slamon, D. and Bookstein, R.** (1997). Comparative mutational analysis of DPC4 (Smad4) in prostatic and colorectal carcinomas. *Oncogene* **15**, 1111-4.
**Maghazachi, A. A. and al-Aoukaty, A.** (1993). Transforming growth factor-beta 1 is chemotactic for interleukin-2-activated natural killer cells. *Nat Immun* **12**, 57-65.
**Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B. et al.** (1995). Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* **268**, 1336-8.
**Massague, J.** (2000). How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* **1**, 169-78.
**Massague, J. and Chen, Y. G.** (2000). Controlling TGF-beta signaling. *Genes Dev* **14**, 627-44.
**Matsui, Y., Halter, S. A., Holt, J. T., Hogan, B. L. and Coffey, R. J.** (1990). Development of mammary hyperplasia and neoplasia in MMTV-TGF alpha transgenic mice. *Cell* **61**, 1147-55.
**Mazar, A. P., Henkin, J. and Goldfarb, R. H.** (1999). The urokinase plasminogen activator system in cancer: implications for tumor angiogenesis and metastasis. *Angiogenesis* **3**, 15-32.
**McDonald, P. P., Fadok, V. A., Bratton, D. and Henson, P. M.** (1999). Transcriptional and translational regulation of inflammatory mediator production by endogenous TGF-beta in macrophages that have ingested apoptotic cells. *J Immunol* **163**, 6164-72.
**Merlo, G. R., Venesio, T., Taverna, D., Marte, B. M., Callahan, R. and Hynes, N. E.** (1994). Growth suppression of normal mammary epithelial cells by wild-type p53. *Oncogene* **9**, 443-53.
**Mieth, M., Boehmer, F. D., Ball, R., Groner, B. and Grosse, R.** (1990). Transforming growth factor-beta inhibits lactogenic hormone induction of beta-casein expression in HC11 mouse mammary epithelial cells. *Growth Factors* **4**, 9-15.
**Miettinen, P. J., Ebner, R., Lopez, A. R. and Derynck, R.** (1994). TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *J Cell Biol* **127**, 2021-36.

**Mikaelian, I., Hovick, M., Silva, K. A., Burzenski, L. M., Shultz, L. D., Ackert-Bicknell, C. L., Cox, G. A. and Sundberg, J. P.** (2006). Expression of terminal differentiation proteins defines stages of mouse mammary gland development. *Vet Pathol* **43**, 36-49.
**Minn, A. J., Gupta, G. P., Siegel, P. M., Bos, P. D., Shu, W., Giri, D. D., Viale, A., Olshen, A. B., Gerald, W. L. and Massague, J.** (2005a). Genes that mediate breast cancer metastasis to lung. *Nature* **436**, 518-24.
**Minn, A. J., Kang, Y., Serganova, I., Gupta, G. P., Giri, D. D., Doubrovin, M., Ponomarev, V., Gerald, W. L., Blasberg, R. and Massague, J.** (2005b). Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. *J Clin Invest* **115**, 44-55.
**Miyoshi, K., Shillingford, J. M., Smith, G. H., Grimm, S. L., Wagner, K. U., Oka, T., Rosen, J. M., Robinson, G. W. and Hennighausen, L.** (2001). Signal transducer and activator of transcription (Stat) 5 controls the proliferation and differentiation of mammary alveolar epithelium. *J Cell Biol* **155**, 531-42.
**Mizoi, T., Ohtani, H., Miyazono, K., Miyazawa, M., Matsuno, S. and Nagura, H.** (1993). Immunoelectron microscopic localization of transforming growth factor beta 1 and latent transforming growth factor beta 1 binding protein in human gastrointestinal carcinomas: qualitative difference between cancer cells and stromal cells. *Cancer Res* **53**, 183-90.
**Moinfar, F., Man, Y. G., Arnould, L., Bratthauer, G. L., Ratschek, M. and Tavassoli, F. A.** (2000). Concurrent and independent genetic alterations in the stromal and epithelial cells of mammary carcinoma: implications for tumorigenesis. *Cancer Res* **60**, 2562-6.
**Moses, H. L., Branum, E. L., Proper, J. A. and Robinson, R. A.** (1981). Transforming growth factor production by chemically transformed cells. *Cancer Res* **41**, 2842-8.
**Moses, H. L., Tucker, R.F., Leof, E.B., Coffey, R.J., Jr., Halper, J., and Shipley, G.D.** (1985). Type beta transforming growth factor is a growth stimulator and a growth inhibitor. *Cancer Cells* **3**, 65-71.
**Moustakas, A. and Heldin, C. H.** (2005). Non-Smad TGF-beta signals. *J Cell Sci* **118**, 3573-84.
**Moustakas, A., Lin, H. Y., Henis, Y. I., Plamondon, J., O'Connor-McCourt, M. D. and Lodish, H. F.** (1993). The transforming growth factor beta receptors types I, II, and III form hetero-oligomeric complexes in the presence of ligand. *J Biol Chem* **268**, 22215-8.
**Mueller, S. O., Clark, J. A., Myers, P. H. and Korach, K. S.** (2002). Mammary gland development in adult mice requires epithelial and stromal estrogen receptor alpha. *Endocrinology* **143**, 2357-65.
**Mukherjee, P., Ginardi, A. R., Madsen, C. S., Tinder, T. L., Jacobs, F., Parker, J., Agrawal, B., Longenecker, B. M. and Gendler, S. J.** (2001). MUC1-specific CTLs are non-functional within a pancreatic tumor microenvironment. *Glycoconj J* **18**, 931-42.
**Mulder, K. M. and Morris, S. L.** (1992). Activation of p21ras by transforming growth factor beta in epithelial cells. *J Biol Chem* **267**, 5029-31.
**Mule, J. J., Schwarz, S. L., Roberts, A. B., Sporn, M. B. and Rosenberg, S. A.** (1988). Transforming growth factor-beta inhibits the in vitro generation of lymphokine-activated killer cells and cytotoxic T cells. *Cancer Immunol Immunother* **26**, 95-100.
**Munger, J. S., Huang, X., Kawakatsu, H., Griffiths, M. J., Dalton, S. L., Wu, J., Pittet, J. F., Kaminski, N., Garat, C., Matthay, M. A. et al.** (1999). The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* **96**, 319-28.
**Muraoka-Cook, R. S., Dumont, N. and Arteaga, C. L.** (2005a). Dual role of transforming growth factor beta in mammary tumorigenesis and metastatic progression. *Clin Cancer Res* **11**, 937s-43s.

**Muraoka-Cook, R. S., Kurokawa, H., Koh, Y., Forbes, J. T., Roebuck, L. R., Barcellos-Hoff, M. H., Moody, S. E., Chodosh, L. A. and Arteaga, C. L.** (2004). Conditional overexpression of active transforming growth factor beta1 in vivo accelerates metastases of transgenic mammary tumors. *Cancer Res* **64**, 9002-11.
**Muraoka-Cook, R. S., Shin, I., Yi, J. Y., Easterly, E., Barcellos-Hoff, M. H., Yingling, J. M., Zent, R. and Arteaga, C. L.** (2005b). Activated type I TGFbeta receptor kinase enhances the survival of mammary epithelial cells and accelerates tumor progression. *Oncogene*.
**Muraoka-Cook, R. S., Shin, I., Yi, J. Y., Easterly, E., Barcellos-Hoff, M. H., Yingling, J. M., Zent, R. and Arteaga, C. L.** (2006). Activated type I TGFbeta receptor kinase enhances the survival of mammary epithelial cells and accelerates tumor progression. *Oncogene* **25**, 3408-23.
**Muraoka, R. S., Dumont, N., Ritter, C. A., Dugger, T. C., Brantley, D. M., Chen, J., Easterly, E., Roebuck, L. R., Ryan, S., Gotwals, P. J. et al.** (2002). Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* **109**, 1551-9.
**Muraoka, R. S., Koh, Y., Roebuck, L. R., Sanders, M. E., Brantley-Sieders, D., Gorska, A. E., Moses, H. L. and Arteaga, C. L.** (2003). Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. *Mol Cell Biol* **23**, 8691-703.
**Nagineni, C. N., Samuel, W., Nagineni, S., Pardhasaradhi, K., Wiggert, B., Detrick, B. and Hooks, J. J.** (2003). Transforming growth factor-beta induces expression of vascular endothelial growth factor in human retinal pigment epithelial cells: involvement of mitogen-activated protein kinases. *J Cell Physiol* **197**, 453-62.
**Nguyen, A. V. and Pollard, J. W.** (2000). Transforming growth factor beta3 induces cell death during the first stage of mammary gland involution. *Development* **127**, 3107-18.
**Oft, M., Akhurst, R. J. and Balmain, A.** (2002). Metastasis is driven by sequential elevation of H-ras and Smad2 levels. *Nat Cell Biol* **4**, 487-94.
**Oft, M., Heider, K. H. and Beug, H.** (1998). TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr Biol* **8**, 1243-52.
**Oft, M., Peli, J., Rudaz, C., Schwarz, H., Beug, H. and Reichmann, E.** (1996). TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev* **10**, 2462-77.
**Olivey, H. E., Mundell, N. A., Austin, A. F. and Barnett, J. V.** (2006). Transforming growth factor-beta stimulates epithelial-mesenchymal transformation in the proepicardium. *Dev Dyn* **235**, 50-9.
**Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carroll, P. R., Tlsty, T. D. and Cunha, G. R.** (1999). Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res* **59**, 5002-11.
**Onder, T. T., Gupta, P. B., Mani, S. A., Yang, J., Lander, E. S. and Weinberg, R. A.** (2008). Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res* **68**, 3645-54.
**Ono, M., Torisu, H., Fukushi, J., Nishie, A. and Kuwano, M.** (1999). Biological implications of macrophage infiltration in human tumor angiogenesis. *Cancer Chemother Pharmacol* **43 Suppl**, S69-71.
**Orimo, A., Gupta, P. B., Sgroi, D. C., Arenzana-Seisdedos, F., Delaunay, T., Naeem, R., Carey, V. J., Richardson, A. L. and Weinberg, R. A.** (2005). Stromal Fibroblasts Present in Invasive Human Breast Carcinomas Promote Tumor Growth and Angiogenesis through Elevated SDF-1/CXCL12 Secretion. *Cell* **121**, 335-48.
**Ortaldo, J. R., Mason, A. T., O'Shea, J. J., Smyth, M. J., Falk, L. A., Kennedy, I. C., Longo, D. L. and Ruscetti, F. W.** (1991). Mechanistic studies of transforming growth factor-beta inhibition of

IL-2-dependent activation of CD3- large granular lymphocyte functions. Regulation of IL-2R beta (p75) signal transduction. *J Immunol* **146**, 3791-8.
**Ossowski, L., Biegel, D. and Reich, E.** (1979). Mammary plasminogen activator: correlation with involution, hormonal modulation and comparison between normal and neoplastic tissue. *Cell* **16**, 929-40.
**Ozdamar, B., Bose, R., Barrios-Rodiles, M., Wang, H. R., Zhang, Y. and Wrana, J. L.** (2005). Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. *Science* **307**, 1603-9.
**Padua, D., Zhang, X. H., Wang, Q., Nadal, C., Gerald, W. L., Gomis, R. R. and Massague, J.** (2008). TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell* **133**, 66-77.
**Pangas, S. A. and Matzuk, M. M.** (2004). Genetic models for transforming growth factor beta superfamily signaling in ovarian follicle development. *Mol Cell Endocrinol* **225**, 83-91.
**Park, J. A., Wang, E., Kurt, R. A., Schluter, S. F., Hersh, E. M. and Akporiaye, E. T.** (1997). Expression of an antisense transforming growth factor-beta1 transgene reduces tumorigenicity of EMT6 mammary tumor cells. *Cancer Gene Ther* **4**, 42-50.
**Pasche, B.** (2001). Role of transforming growth factor beta in cancer. *J Cell Physiol* **186**, 153-68.
**Peng, S. B., Yan, L., Xia, X., Watkins, S. A., Brooks, H. B., Beight, D., Herron, D. K., Jones, M. L., Lampe, J. W., McMillen, W. T. et al.** (2005). Kinetic characterization of novel pyrazole TGF-beta receptor I kinase inhibitors and their blockade of the epithelial-mesenchymal transition. *Biochemistry* **44**, 2293-304.
**Pepper, M. S.** (1997). Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth Factor Rev* **8**, 21-43.
**Pepper, M. S., Vassalli, J. D., Orci, L. and Montesano, R.** (1993). Biphasic effect of transforming growth factor-beta 1 on in vitro angiogenesis. *Exp Cell Res* **204**, 356-63.
**Perlman, R., Schiemann, W. P., Brooks, M. W., Lodish, H. F. and Weinberg, R. A.** (2001). TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. *Nat Cell Biol* **3**, 708-14.
**Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A. et al.** (2000). Molecular portraits of human breast tumours. *Nature* **406**, 747-52.
**Petritsch, C., Beug, H., Balmain, A. and Oft, M.** (2000). TGF-beta inhibits p70 S6 kinase via protein phosphatase 2A to induce G(1) arrest. *Genes Dev* **14**, 3093-101.
**Pierce, D. F., Jr., Gorska, A. E., Chytil, A., Meise, K. S., Page, D. L., Coffey, R. J., Jr. and Moses, H. L.** (1995). Mammary tumor suppression by transforming growth factor beta 1 transgene expression. *Proc Natl Acad Sci U S A* **92**, 4254-8.
**Pierce, D. F., Jr., Johnson, M. D., Matsui, Y., Robinson, S. D., Gold, L. I., Purchio, A. F., Daniel, C. W., Hogan, B. L. and Moses, H. L.** (1993). Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-beta 1. *Genes Dev* **7**, 2308-17.
**Pietenpol, J. A., Holt, J. T., Stein, R. W. and Moses, H. L.** (1990). Transforming growth factor beta 1 suppression of c-myc gene transcription: role in inhibition of keratinocyte proliferation. *Proc Natl Acad Sci U S A* **87**, 3758-62.
**Pommier, Y., Sordet, O., Antony, S., Hayward, R. L. and Kohn, K. W.** (2004). Apoptosis defects and chemotherapy resistance: molecular interaction maps and networks. *Oncogene* **23**, 2934-49.

**Porcile, C., Bajetto, A., Barbero, S., Pirani, P. and Schettini, G.** (2004). CXCR4 activation induces epidermal growth factor receptor transactivation in an ovarian cancer cell line. *Ann N Y Acad Sci* **1030**, 162-9.
**Postlethwaite, A. E., Keski-Oja, J., Moses, H. L. and Kang, A. H.** (1987). Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta. *J Exp Med* **165**, 251-6.
**Proetzel, G., Pawlowski, S. A., Wiles, M. V., Yin, M., Boivin, G. P., Howles, P. N., Ding, J., Ferguson, M. W. and Doetschman, T.** (1995). Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat Genet* **11**, 409-14.
**Rajagopalan, H., Nowak, M. A., Vogelstein, B. and Lengauer, C.** (2003). The significance of unstable chromosomes in colorectal cancer. *Nat Rev Cancer* **3**, 695-701.
**Rajan, J. V., Wang, M., Marquis, S. T. and Chodosh, L. A.** (1996). Brca2 is coordinately regulated with Brca1 during proliferation and differentiation in mammary epithelial cells. *Proc Natl Acad Sci U S A* **93**, 13078-83.
**Reibman, J., Meixler, S., Lee, T. C., Gold, L. I., Cronstein, B. N., Haines, K. A., Kolasinski, S. L. and Weissmann, G.** (1991). Transforming growth factor beta 1, a potent chemoattractant for human neutrophils, bypasses classic signal-transduction pathways. *Proc Natl Acad Sci U S A* **88**, 6805-9.
**Ren, Y., Chan, H. M., Li, Z., Lin, C., Nicholls, J., Chen, C. F., Lee, P. Y., Lui, V., Bacher, M. and Tam, P. K.** (2004). Upregulation of macrophage migration inhibitory factor contributes to induced N-Myc expression by the activation of ERK signaling pathway and increased expression of interleukin-8 and VEGF in neuroblastoma. *Oncogene* **23**, 4146-54.
**Riedl, S. J. and Salvesen, G. S.** (2007). The apoptosome: signalling platform of cell death. *Nat Rev Mol Cell Biol* **8**, 405-13.
**Roberts, A. B., Anzano, M. A., Lamb, L. C., Smith, J. M. and Sporn, M. B.** (1981). New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. *Proc Natl Acad Sci U S A* **78**, 5339-43.
**Roberts, A. B. and Wakefield, L. M.** (2003). The two faces of transforming growth factor beta in carcinogenesis. *Proc Natl Acad Sci U S A* **100**, 8621-3.
**Robinson, S. D., Roberts, A. B. and Daniel, C. W.** (1993). TGF beta suppresses casein synthesis in mouse mammary explants and may play a role in controlling milk levels during pregnancy. *J Cell Biol* **120**, 245-51.
**Robinson, S. D., Silberstein, G. B., Roberts, A. B., Flanders, K. C. and Daniel, C. W.** (1991). Regulated expression and growth inhibitory effects of transforming growth factor-beta isoforms in mouse mammary gland development. *Development* **113**, 867-78.
**Ruddy, M. J., Shen, F., Smith, J. B., Sharma, A. and Gaffen, S. L.** (2004). Interleukin-17 regulates expression of the CXC chemokine LIX/CXCL5 in osteoblasts: implications for inflammation and neutrophil recruitment. *J Leukoc Biol* **76**, 135-44.
**Ruzek, M. C., Hawes, M., Pratt, B., McPherson, J., Ledbetter, S., Richards, S. M. and Garman, R. D.** (2003). Minimal effects on immune parameters following chronic anti-TGF-beta monoclonal antibody administration to normal mice. *Immunopharmacol Immunotoxicol* **25**, 235-57.
**Sakakura, T., Sakagami, Y. and Nishizuka, Y.** (1981). Accelerated mammary cancer development by fetal salivary mesenchyma isografted to adult mouse mammary epithelium. *J Natl Cancer Inst* **66**, 953-9.
**San Francisco, I. F., DeWolf, W. C., Peehl, D. M. and Olumi, A. F.** (2004). Expression of transforming growth factor-beta 1 and growth in soft agar differentiate prostate carcinoma-associated fibroblasts from normal prostate fibroblasts. *Int J Cancer* **112**, 213-8.

**Scaffidi, A. K., Petrovic, N., Moodley, Y. P., Fogel-Petrovic, M., Kroeger, K. M., Seeber, R. M., Eidne, K. A., Thompson, P. J. and Knight, D. A.** (2004). alpha(v)beta(3) Integrin interacts with the transforming growth factor beta (TGFbeta) type II receptor to potentiate the proliferative effects of TGFbeta1 in living human lung fibroblasts. *J Biol Chem* **279**, 37726-33.
**Schlingensiepen, K. H., Schlingensiepen, R., Steinbrecher, A., Hau, P., Bogdahn, U., Fischer-Blass, B. and Jachimczak, P.** (2005a). Targeted tumor therapy with the TGF-beta2 antisense compound AP 12009. *Cytokine Growth Factor Rev*.
**Schlingensiepen, R., Goldbrunner, M., Szyrach, M. N., Stauder, G., Jachimczak, P., Bogdahn, U., Schulmeyer, F., Hau, P. and Schlingensiepen, K. H.** (2005b). Intracerebral and intrathecal infusion of the TGF-beta2-specific antisense phosphorothioate oligonucleotide AP 12009 in rabbits and primates: Toxicology and Safety. *Oligonucleotides* **15**, 94-104.
**Schmeisser, A., Garlichs, C. D., Zhang, H., Eskafi, S., Graffy, C., Ludwig, J., Strasser, R. H. and Daniel, W. G.** (2001). Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel under angiogenic conditions. *Cardiovasc Res* **49**, 671-80.
**Schmeisser, A. and Strasser, R. H.** (2002). Phenotypic overlap between hematopoietic cells with suggested angioblastic potential and vascular endothelial cells. *J Hematother Stem Cell Res* **11**, 69-79.
**Schoppmann, S. F., Birner, P., Stockl, J., Kalt, R., Ullrich, R., Caucig, C., Kriehuber, E., Nagy, K., Alitalo, K. and Kerjaschki, D.** (2002). Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *Am J Pathol* **161**, 947-56.
**Schuster, N. and Krieglstein, K.** (2002). Mechanisms of TGF-beta-mediated apoptosis. *Cell Tissue Res* **307**, 1-14.
**Schutte, M., Hruban, R. H., Hedrick, L., Cho, K. R., Nadasdy, G. M., Weinstein, C. L., Bova, G. S., Isaacs, W. B., Cairns, P., Nawroz, H. et al.** (1996). DPC4 gene in various tumor types. *Cancer Res* **56**, 2527-30.
**Seitz, S., Wassmuth, P., Plaschke, J., Schackert, H. K., Karsten, U., Santibanez-Koref, M. F., Schlag, P. M. and Scherneck, S.** (2003). Identification of microsatellite instability and mismatch repair gene mutations in breast cancer cell lines. *Genes Chromosomes Cancer* **37**, 29-35.
**Senoo, M., Pinto, F., Crum, C. P. and McKeon, F.** (2007). p63 Is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* **129**, 523-36.
**Serafini, P., De Santo, C., Marigo, I., Cingarlini, S., Dolcetti, L., Gallina, G., Zanovello, P. and Bronte, V.** (2004). Derangement of immune responses by myeloid suppressor cells. *Cancer Immunol Immunother* **53**, 64-72.
**Shackleton, M., Vaillant, F., Simpson, K. J., Stingl, J., Smyth, G. K., Asselin-Labat, M. L., Wu, L., Lindeman, G. J. and Visvader, J. E.** (2006). Generation of a functional mammary gland from a single stem cell. *Nature* **439**, 84-8.
**Shah, A. H., Tabayoyong, W. B., Kundu, S. D., Kim, S. J., Van Parijs, L., Liu, V. C., Kwon, E., Greenberg, N. M. and Lee, C.** (2002). Suppression of tumor metastasis by blockade of transforming growth factor beta signaling in bone marrow cells through a retroviral-mediated gene therapy in mice. *Cancer Res* **62**, 7135-8.
**Sheffield, L. G. and Kotolski, L. C.** (1992). Prolactin inhibits programmed cell death during mammary gland involution. *Faseb J* **6**.
**Shi, Y. and Massague, J.** (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.

**Shibuya, H., Yamaguchi, K., Shirakabe, K., Tonegawa, A., Gotoh, Y., Ueno, N., Irie, K., Nishida, E. and Matsumoto, K.** (1996). TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. *Science* **272**, 1179-82.
**Shih, S. C. and Claffey, K. P.** (2001). Role of AP-1 and HIF-1 transcription factors in TGF-beta activation of VEGF expression. *Growth Factors* **19**, 19-34.
**Shillingford, J. M., Miyoshi, K., Robinson, G. W., Bierie, B., Cao, Y., Karin, M. and Hennighausen, L.** (2003). Proteotyping of mammary tissue from transgenic and gene knockout mice with immunohistochemical markers: a tool to define developmental lesions. *J Histochem Cytochem* **51**, 555-65.
**Shipitsin, M., Campbell, L. L., Argani, P., Weremowicz, S., Bloushtain-Qimron, N., Yao, J., Nikolskaya, T., Serebryiskaya, T., Beroukhim, R., Hu, M. et al.** (2007). Molecular definition of breast tumor heterogeneity. *Cancer Cell* **11**, 259-73.
**Shull, M. M., Ormsby, I., Kier, A. B., Pawlowski, S., Diebold, R. J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D. et al.** (1992). Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* **359**, 693-9.
**Siegel, P. M. and Massague, J.** (2003). Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* **3**, 807-21.
**Siegel, P. M., Shu, W., Cardiff, R. D., Muller, W. J. and Massague, J.** (2003). Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc Natl Acad Sci U S A* **100**, 8430-5.
**Siepl, C., Malipiero, U. V. and Fontana, A.** (1991). Transforming growth factor-beta (TGF-beta)-resistant helper T lymphocyte clones show a concomitant loss of all three types of TGF-beta receptors. *J Immunol* **146**, 3063-7.
**Silberstein, G. B. and Daniel, C. W.** (1987). Reversible inhibition of mammary gland growth by transforming growth factor-beta. *Science* **237**, 291-3.
**Smyth, M. J., Teng, M. W., Swann, J., Kyparissoudis, K., Godfrey, D. I. and Hayakawa, Y.** (2006). CD4+CD25+ T Regulatory Cells Suppress NK Cell-Mediated Immunotherapy of Cancer. *J Immunol* **176**, 1582-7.
**Soriano, P.** (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* **21**, 70-1.
**Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S. et al.** (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* **98**, 10869-74.
**Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J. S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S. et al.** (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A* **100**, 8418-23.
**Stein, T., Morris, J. S., Davies, C. R., Weber-Hall, S. J., Duffy, M. A., Heath, V. J., Bell, A. K., Ferrier, R. K., Sandilands, G. P. and Gusterson, B. A.** (2004). Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. *Breast Cancer Res* **6**, R75-91.
**Streuli, C. H., Schmidhauser, C., Kobrin, M., Bissell, M. J. and Derynck, R.** (1993). Extracellular matrix regulates expression of the TGF-beta 1 gene. *J Cell Biol* **120**, 253-60.
**Subramanian, G., Schwarz, R. E., Higgins, L., McEnroe, G., Chakravarty, S., Dugar, S. and Reiss, M.** (2004). Targeting endogenous transforming growth factor beta receptor signaling in SMAD4-deficient human pancreatic carcinoma cells inhibits their invasive phenotype1. *Cancer Res* **64**, 5200-11.

**Sudlow, A. W., Wilde, C. J. and Burgoyne, R. D.** (1994). Transforming growth factor-beta 1 inhibits casein secretion from differentiating mammary-gland explants but not from lactating mammary cells. *Biochem J* **304 ( Pt 2)**, 333-6.
**Sugimoto, H., Mundel, T. M., Kieran, M. W. and Kalluri, R.** (2006). Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer Biol Ther* **5**, 1640-6.
**Sun, B., Nishihira, J., Yoshiki, T., Kondo, M., Sato, Y., Sasaki, F. and Todo, S.** (2005). Macrophage migration inhibitory factor promotes tumor invasion and metastasis via the Rho-dependent pathway. *Clin Cancer Res* **11**, 1050-8.
**Sun, L., Wu, G., Willson, J. K., Zborowska, E., Yang, J., Rajkarunanayake, I., Wang, J., Gentry, L. E., Wang, X. F. and Brattain, M. G.** (1994). Expression of transforming growth factor beta type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. *J Biol Chem* **269**, 26449-55.
**Sun, Y., Cheng, Z., Ma, L. and Pei, G.** (2002). Beta-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. *J Biol Chem* **277**, 49212-9.
**Takagi, Y., Koumura, H., Futamura, M., Aoki, S., Ymaguchi, K., Kida, H., Tanemura, H., Shimokawa, K. and Saji, S.** (1998). Somatic alterations of the SMAD-2 gene in human colorectal cancers. *Br J Cancer* **78**, 1152-5.
**Takahashi, N., Nishihira, J., Sato, Y., Kondo, M., Ogawa, H., Ohshima, T., Une, Y. and Todo, S.** (1998). Involvement of macrophage migration inhibitory factor (MIF) in the mechanism of tumor cell growth. *Mol Med* **4**, 707-14.
**Takaku, K., Oshima, M., Miyoshi, H., Matsui, M., Seldin, M. F. and Taketo, M. M.** (1998). Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. *Cell* **92**, 645-56.
**Takaku, K., Wrana, J. L., Robertson, E. J. and Taketo, M. M.** (2002). No effects of Smad2 (madh2) null mutation on malignant progression of intestinal polyps in Apc(delta716) knockout mice. *Cancer Res* **62**, 4558-61.
**Takenoshita, S., Mogi, A., Tani, M., Osawa, H., Sunaga, H., Kakegawa, H., Yanagita, Y., Koida, T., Kimura, M., Fujita, K. I. et al.** (1998). Absence of mutations in the analysis of coding sequences of the entire transforming growth factor-beta type II receptor gene in sporadic human breast cancers. *Oncol Rep* **5**, 367-71.
**Taketo, M. M. and Takaku, K.** (2000). Gastrointestinal tumorigenesis in Smad4 (Dpc4) mutant mice. *Hum Cell* **13**, 85-95.
**Tang, B., Bottinger, E. P., Jakowlew, S. B., Bagnall, K. M., Mariano, J., Anver, M. R., Letterio, J. J. and Wakefield, L. M.** (1998). Transforming growth factor-beta1 is a new form of tumor suppressor with true haploid insufficiency. *Nat Med* **4**, 802-7.
**Tang, B., Vu, M., Booker, T., Santner, S. J., Miller, F. R., Anver, M. R. and Wakefield, L. M.** (2003). TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest* **112**, 1116-24.
**Thangaraju, M., Rudelius, M., Bierie, B., Raffeld, M., Sharan, S., Hennighausen, L., Huang, A. M. and Sterneck, E.** (2005). C/EBPdelta is a crucial regulator of pro-apoptotic gene expression during mammary gland involution. *Development* **132**, 4675-85.
**Thiagalingam, S., Lengauer, C., Leach, F. S., Schutte, M., Hahn, S. A., Overhauser, J., Willson, J. K., Markowitz, S., Hamilton, S. R., Kern, S. E. et al.** (1996). Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. *Nat Genet* **13**, 343-6.
**Thomas, D. A. and Massague, J.** (2005). TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* **8**, 369-80.

**Thompson, J. E., Vaughan, T. J., Williams, A. J., Wilton, J., Johnson, K. S., Bacon, L., Green, J. A., Field, R., Ruddock, S., Martins, M. et al.** (1999). A fully human antibody neutralising biologically active human TGFbeta2 for use in therapy. *J Immunol Methods* **227**, 17-29.
**Tian, F., DaCosta Byfield, S., Parks, W. T., Yoo, S., Felici, A., Tang, B., Piek, E., Wakefield, L. M. and Roberts, A. B.** (2003). Reduction in Smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. *Cancer Res* **63**, 8284-92.
**Tojo, M., Hamashima, Y., Hanyu, A., Kajimoto, T., Saitoh, M., Miyazono, K., Node, M. and Imamura, T.** (2005). The ALK-5 inhibitor A-83-01 inhibits Smad signaling and epithelial-to-mesenchymal transition by transforming growth factor-beta. *Cancer Sci* **96**, 791-800.
**Tokuda, H., Hatakeyama, D., Akamatsu, S., Tanabe, K., Yoshida, M., Shibata, T. and Kozawa, O.** (2003). Involvement of MAP kinases in TGF-beta-stimulated vascular endothelial growth factor synthesis in osteoblasts. *Arch Biochem Biophys* **415**, 117-25.
**Topper, Y. J. and Freeman, C. S.** (1980). Multiple hormone interactions in the developmental biology of the mammary gland. *Physiol Rev* **60**, 1049-106.
**Torisu, H., Ono, M., Kiryu, H., Furue, M., Ohmoto, Y., Nakayama, J., Nishioka, Y., Sone, S. and Kuwano, M.** (2000). Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: possible involvement of TNFalpha and IL-1alpha. *Int J Cancer* **85**, 182-8.
**Tsushima, H., Kawata, S., Tamura, S., Ito, N., Shirai, Y., Kiso, S., Imai, Y., Shimomukai, H., Nomura, Y., Matsuda, Y. et al.** (1996). High levels of transforming growth factor beta 1 in patients with colorectal cancer: association with disease progression. *Gastroenterology* **110**, 375-82.
**Tucker, R. F., Shipley, G. D., Moses, H. L. and Holley, R. W.** (1984). Growth inhibitor from BSC-1 cells closely related to platelet type beta transforming growth factor. *Science* **226**, 705-7.
**Ueda, Y., Wang, S., Dumont, N., Yi, J. Y., Koh, Y. and Arteaga, C. L.** (2004). Overexpression of HER2 (erbB2) in human breast epithelial cells unmasks transforming growth factor beta-induced cell motility. *J Biol Chem* **279**, 24505-13.
**Uhl, M., Aulwurm, S., Wischhusen, J., Weiler, M., Ma, J. Y., Almirez, R., Mangadu, R., Liu, Y. W., Platten, M., Herrlinger, U. et al.** (2004). SD-208, a novel transforming growth factor beta receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells in vitro and in vivo. *Cancer Res* **64**, 7954-61.
**van 't Veer, L. J., Dai, H., van de Vijver, M. J., He, Y. D., Hart, A. A., Mao, M., Peterse, H. L., van der Kooy, K., Marton, M. J., Witteveen, A. T. et al.** (2002). Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415**, 530-6.
**van de Rijn, M., Perou, C. M., Tibshirani, R., Haas, P., Kallioniemi, O., Kononen, J., Torhorst, J., Sauter, G., Zuber, M., Kochli, O. R. et al.** (2002). Expression of cytokeratins 17 and 5 identifies a group of breast carcinomas with poor clinical outcome. *Am J Pathol* **161**, 1991-6.
**van de Vijver, M. J., He, Y. D., van't Veer, L. J., Dai, H., Hart, A. A., Voskuil, D. W., Schreiber, G. J., Peterse, J. L., Roberts, C., Marton, M. J. et al.** (2002). A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* **347**, 1999-2009.
**Vinals, F. and Pouyssegur, J.** (2001). Transforming growth factor beta1 (TGF-beta1) promotes endothelial cell survival during in vitro angiogenesis via an autocrine mechanism implicating TGF-alpha signaling. *Mol Cell Biol* **21**, 7218-30.
**Vogelstein, B. and Kinzler, K. W.** (2004). Cancer genes and the pathways they control. *Nat Med* **10**, 789-99.
**Wagner, K. U., McAllister, K., Ward, T., Davis, B., Wiseman, R. and Hennighausen, L.** (2001). Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. *Transgenic Res* **10**, 545-53.

**Wagner, K. U., Wall, R. J., St-Onge, L., Gruss, P., Wynshaw-Boris, A., Garrett, L., Li, M., Furth, P. A. and Hennighausen, L.** (1997). Cre-mediated gene deletion in the mammary gland. *Nucleic Acids Res* **25**, 4323-30.
**Wahl, S. M., Swisher, J., McCartney-Francis, N. and Chen, W.** (2004). TGF-beta: the perpetrator of immune suppression by regulatory T cells and suicidal T cells. *J Leukoc Biol* **76**, 15-24.
**Wakefield, L. M. and Sporn, M. B.** (1990). Suppression of carcinogenesis: a role for TGF-beta and related molecules in prevention of cancer. *Immunol Ser* **51**, 217-43.
**Walia, B., Wang, L., Merlin, D. and Sitaraman, S. V.** (2003). TGF-beta down-regulates IL-6 signaling in intestinal epithelial cells: critical role of SMAD-2. *Faseb J* **17**, 2130-2.
**Wallick, S. C., Figari, I. S., Morris, R. E., Levinson, A. D. and Palladino, M. A.** (1990). Immunoregulatory role of transforming growth factor beta (TGF-beta) in development of killer cells: comparison of active and latent TGF-beta 1. *J Exp Med* **172**, 1777-84.
**Wang, J., Guan, E., Roderiquez, G., Calvert, V., Alvarez, R. and Norcross, M. A.** (2001). Role of tyrosine phosphorylation in ligand-independent sequestration of CXCR4 in human primary monocytes-macrophages. *J Biol Chem* **276**, 49236-43.
**Wang, W., Goswami, S., Sahai, E., Wyckoff, J. B., Segall, J. E. and Condeelis, J. S.** (2005a). Tumor cells caught in the act of invading: their strategy for enhanced cell motility. *Trends Cell Biol* **15**, 138-45.
**Wang, X. F., Lin, H. Y., Ng-Eaton, E., Downward, J., Lodish, H. F. and Weinberg, R. A.** (1991). Expression cloning and characterization of the TGF-beta type III receptor. *Cell* **67**, 797-805.
**Wang, X. J., Greenhalgh, D. A., Bickenbach, J. R., Jiang, A., Bundman, D. S., Krieg, T., Derynck, R. and Roop, D. R.** (1997). Expression of a dominant-negative type II transforming growth factor beta (TGF-beta) receptor in the epidermis of transgenic mice blocks TGF-beta-mediated growth inhibition. *Proc Natl Acad Sci U S A* **94**, 2386-91.
**Wang, X. J., Liefer, K. M., Tsai, S., O'Malley, B. W. and Roop, D. R.** (1999). Development of gene-switch transgenic mice that inducibly express transforming growth factor beta1 in the epidermis. *Proc Natl Acad Sci U S A* **96**, 8483-8.
**Wang, Y., Klijn, J. G., Zhang, Y., Sieuwerts, A. M., Look, M. P., Yang, F., Talantov, D., Timmermans, M., Meijer-van Gelder, M. E., Yu, J. et al.** (2005b). Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* **365**, 671-9.
**Warner, K. A., Miyazawa, M., Cordeiro, M. M., Love, W. J., Pinsky, M. S., Neiva, K. G., Spalding, A. C. and Nor, J. E.** (2008). Endothelial cells enhance tumor cell invasion through a crosstalk mediated by CXC chemokine signaling. *Neoplasia* **10**, 131-9.
**Weeks, B. H., He, W., Olson, K. L. and Wang, X. J.** (2001). Inducible expression of transforming growth factor beta1 in papillomas causes rapid metastasis. *Cancer Res* **61**, 7435-43.
**Welch, D. R., Fabra, A. and Nakajima, M.** (1990). Transforming growth factor beta stimulates mammary adenocarcinoma cell invasion and metastatic potential. *Proc Natl Acad Sci U S A* **87**, 7678-82.
**Wikstrom, P., Stattin, P., Franck-Lissbrant, I., Damber, J. E. and Bergh, A.** (1998). Transforming growth factor beta1 is associated with angiogenesis, metastasis, and poor clinical outcome in prostate cancer. *Prostate* **37**, 19-29.
**Wilkes, M. C., Murphy, S. J., Garamszegi, N. and Leof, E. B.** (2003). Cell-type-specific activation of PAK2 by transforming growth factor beta independent of Smad2 and Smad3. *Mol Cell Biol* **23**, 8878-89.
**Witham, T. F., Villa, L., Yang, T., Pollack, I. F., Okada, H., Robbins, P. D. and Chambers, W. H.** (2003). Expression of a soluble transforming growth factor-beta (TGFbeta) receptor reduces tumorigenicity by regulating natural killer (NK) cell activity against 9L gliosarcoma in vivo. *J Neurooncol* **64**, 63-9.

**Wrana, J. L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X. F. and Massague, J.** (1992). TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* **71**, 1003-14.
**Xiao, Y. Q., Malcolm, K., Worthen, G. S., Gardai, S., Schiemann, W. P., Fadok, V. A., Bratton, D. L. and Henson, P. M.** (2002). Cross-talk between ERK and p38 MAPK mediates selective suppression of pro-inflammatory cytokines by transforming growth factor-beta. *J Biol Chem* **277**, 14884-93.
**Xie, L., Law, B. K., Aakre, M. E., Edgerton, M., Shyr, Y., Bhowmick, N. A. and Moses, H. L.** (2003). Transforming growth factor beta-regulated gene expression in a mouse mammary gland epithelial cell line. *Breast Cancer Res* **5**, R187-98.
**Xu, G., Chakraborty, C. and Lala, P. K.** (2003). Reconstitution of Smad3 restores TGF-beta response of tissue inhibitor of metalloprotease-1 upregulation in human choriocarcinoma cells. *Biochem Biophys Res Commun* **300**, 383-90.
**Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E. and Matsumoto, K.** (1995). Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. *Science* **270**, 2008-11.
**Yang, D. C., Elliott, R. L. and Head, J. F.** (2002a). Gene targets of antisense therapies in breast cancer. *Expert Opin Ther Targets* **6**, 375-85.
**Yang, J., Mani, S. A., Donaher, J. L., Ramaswamy, S., Itzykson, R. A., Come, C., Savagner, P., Gitelman, I., Richardson, A. and Weinberg, R. A.** (2004a). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* **117**, 927-39.
**Yang, L., Debusk, L. M., Fukuda, K., Fingleton, B., Green-Jarvis, B., Shyr, Y., Matrisian, L. M., Carbone, D. P. and Lin, P. C.** (2004b). Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* **6**, 409-21.
**Yang, L., Huang, J., Ren, X., Gorska, A. E., Chytil, A., Aakre, M., Carbone, D. P., Matrisian, L., Richmond, A., Lin, P. C. et al.** (2008). Abrogation of TGFb signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer Cell* **In-Press**.
**Yang, Y. A., Dukhanina, O., Tang, B., Mamura, M., Letterio, J. J., MacGregor, J., Patel, S. C., Khozin, S., Liu, Z. Y., Green, J. et al.** (2002b). Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* **109**, 1607-15.
**Yang, Y. A., Tang, B., Robinson, G., Hennighausen, L., Brodie, S. G., Deng, C. X. and Wakefield, L. M.** (2002c). Smad3 in the mammary epithelium has a nonredundant role in the induction of apoptosis, but not in the regulation of proliferation or differentiation by transforming growth factor-beta. *Cell Growth Differ* **13**, 123-30.
**Yi, J. Y., Shin, I. and Arteaga, C. L.** (2005). Type I transforming growth factor beta receptor binds to and activates phosphatidylinositol 3-kinase. *J Biol Chem* **280**, 10870-6.
**Yingling, J. M., Blanchard, K. L. and Sawyer, J. S.** (2004). Development of TGF-beta signalling inhibitors for cancer therapy. *Nat Rev Drug Discov* **3**, 1011-22.
**Yoshinaga, K., Mimori, K., Yamashita, K., Utsunomiya, T., Inoue, H. and Mori, M.** (2003). Clinical significance of the expression of activin A in esophageal carcinoma. *Int J Oncol* **22**, 75-80.
**Young, M. R. and Lathers, D. M.** (1999). Myeloid progenitor cells mediate immune suppression in patients with head and neck cancers. *Int J Immunopharmacol* **21**, 241-52.
**Young, M. R., Wright, M. A., Matthews, J. P., Malik, I. and Prechel, M.** (1996). Suppression of T cell proliferation by tumor-induced granulocyte-macrophage progenitor cells producing transforming growth factor-beta and nitric oxide. *J Immunol* **156**, 1916-22.
**Yu, Q. and Stamenkovic, I.** (2000). Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* **14**, 163-76.

**Zavadil, J., Bitzer, M., Liang, D., Yang, Y. C., Massimi, A., Kneitz, S., Piek, E. and Bottinger, E. P.** (2001). Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. *Proc Natl Acad Sci U S A* **98**, 6686-91.

**Zhang, L., Wang, W., Hayashi, Y., Jester, J. V., Birk, D. E., Gao, M., Liu, C. Y., Kao, W. W., Karin, M. and Xia, Y.** (2003). A role for MEK kinase 1 in TGF-beta/activin-induced epithelium movement and embryonic eyelid closure. *Embo J* **22**, 4443-54.

**Zhang, Q., Yang, X., Pins, M., Javonovic, B., Kuzel, T., Kim, S. J., Parijs, L. V., Greenberg, N. M., Liu, V., Guo, Y. et al.** (2005). Adoptive transfer of tumor-reactive transforming growth factor-beta-insensitive CD8+ T cells: eradication of autologous mouse prostate cancer. *Cancer Res* **65**, 1761-9.

**Zhao, L., Hart, S., Cheng, J., Melenhorst, J. J., Bierie, B., Ernst, M., Stewart, C., Schaper, F., Heinrich, P. C., Ullrich, A. et al.** (2004). Mammary gland remodeling depends on gp130 signaling through Stat3 and MAPK. *J Biol Chem* **279**, 44093-100.

**Zhao, L., Melenhorst, J. J. and Hennighausen, L.** (2002). Loss of interleukin 6 results in delayed mammary gland involution: a possible role for mitogen-activated protein kinase and not signal transducer and activator of transcription 3. *Mol Endocrinol* **16**, 2902-12.

**Zhao, S., Venkatasubbarao, K., Lazor, J. W., Sperry, J., Jin, C., Cao, L. and Freeman, J. W.** (2008). Inhibition of STAT3 Tyr705 phosphorylation by Smad4 suppresses transforming growth factor beta-mediated invasion and metastasis in pancreatic cancer cells. *Cancer Res* **68**, 4221-8.

**Ziv, E., Cauley, J., Morin, P. A., Saiz, R. and Browner, W. S.** (2001). Association between the T29->C polymorphism in the transforming growth factor beta1 gene and breast cancer among elderly white women: The Study of Osteoporotic Fractures. *Jama* **285**, 2859-63.