CELL TYPE SPECIFIC TGF-β INDUCED ACTIVATION OF ERK1/2

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Cell Type Specific TGF-β Induced Activation of Erk1/2

By

©Christopher Michael Hough

A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science

Faculty of Medicine
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ABSTRACT

The Transforming Growth Factor-Beta (TGF-β) family is a collection of structurally related peptides. These growth factors are involved in a variety of cellular processes such as apoptosis, differentiation, and proliferation. TGF-β binding to a Serine/Threonine kinase receptor complex causes the recruitment and subsequent activation of transcription factors known as Smad2 and Smad3. These proteins then translocate into the nucleus and either negatively or positively regulate gene expression. TGF-β acts in a cell type specific manner; cellular proliferation is induced in mesenchymal cells and inhibited in epithelial cells. In this study, we define a novel Smad-independent pathway leading to the phosphorylation of extracellular signal regulated kinase (Erk) in a cell type dependent fashion. Erk activation is seen in mesenchymal cells, but not in cells of epithelial origin. Phosphotidylinositol 3-Kinase (PI3K) appears to function upstream of Erk by activating cdc42/Rac1 and subsequently p21-Activated Kinase2 (PAK2). PAK2 activity was shown to be integral to Erk activation through the phosphorylation of c-Raf at Ser338, a site important for c-Raf activity. The MEK kinases were found to act directly upstream of Erk as the presence of U0126 abolishes TGF-β induced Erk phosphorylation. Furthermore, Erk activity is critical in TGF-β induced fibroblast proliferation, likely through its interactions with transcription factors such as Smads and c-myc. Moreover, we have shown a direct interaction between Erk and Smads during TGF-β stimulation. Phosphorylation of Serine residues 245, 250, and 255 within the linker region of Smad2 was observed after induction of Erk. Interestingly, this

phosphorylation event was localized within the nucleus. Together, this data shows a new signaling pathway utilized by TGF-β receptors that interacts with and regulates the classical Smad signaling pathway and TGF-β induced proliferation in fibroblasts.

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Abbreviations

Cdk	Cyclin-Dependent Kinase
DMEM	Dulbecco's Modified Eagle's Medium
EGF	Epidermal Growth Factor
Erk	Extracellular Signal Regulated Kinase
FCS	Fetal Calf Serum
GFP	Green Fluorescent Protein
NBCS	Newborn Calf Serum
PAK	p21-Activated Kinase
PMSF	Phenylmethylsulfonyl fluoride
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Smad	*Sma* and *mad*-related protein
TEMED	tetramethylethylenediamine
TGF-β	Transforming Growth Factor-β
TGFβR	Transforming Growth Factor-β Receptor

CHAPTER 1 - Literature Review

TGF-β/Smad Signaling

1.1.1 TGF-β Family

The Transforming Growth Factor-β family consists of a large number of structurally related proteins mediating a wide variety of biological effects including proliferation, differentiation, and cell death (Ignotz and Massague, 1985;Roberts *et al.*, 1985;Jetten *et al.*, 1986;Rotello *et al.*, 1991;Oberhammer *et al.*, 1992;Yanagihara and Tsumuraya, 1992). Two subfamilies are defined within this group of cytokines; the first being the TGF-β/Activin/Nodal subfamily, the other being the BMP (Bone Morphogenic Protein)/GDF (Growth and Differentiation Factor)/MIS (Muellerian inhibiting substance) subfamily (Massague *et al.*, 2000). The BMPs together form the largest group within the TGF-β family and are known for their role in osteogenesis (Wozney *et al.*, 1988). TGF-β and Activin are involved in the later stages of embryogenesis and in the mature organism controlling immune regulation and tissue repair (Smith *et al.*, 1988;van den Eijnden-Van Raaij AJ *et al.*, 1990;Massague *et al.*, 2000).

The TGF-β subset includes three isoforms in mammals, TGF-β1, β2, and β3. TGF-β1 is the most abundant form and controls and regulates a wide array of developmental and immune processes (Letterio and Roberts, 1998). TGF-β1 has an important role during development and for regulation of immune cell proliferation. TGF-β1 null mice develop normally for the first 2 weeks post-partum, after which massive infiltration of macrophages and lymphocytes into the heart and lungs occurs. Death occurs within 3-4 weeks of age (Kulkarni *et al.*, 1993). TGF-β2 is important in a number

of developmental pathways. As such, β2 knockout mice exhibit a number of tissue abnormalities including heart, skeletal, and craniofacial defects (Sanford *et al.*, 1997), while TGF-β3 deficient mice show defects in lung and palate morphogenesis, as well as early age mortality (Barton *et al.*, 1988;Ten Dijke *et al.*, 1988).

All three isoforms are secreted as latent complexes (Pircher *et al.*, 1984;Lawrence *et al.*, 1984;Lawrence *et al.*, 1985;Pircher *et al.*, 1986;Brown *et al.*, 1990). Since the half-life of mature TGF-β is only a few minutes *in situ*, the latency of the molecule provides stability and a ready source of the growth factor for the organism (Wakefield *et al.*, 1990). TGF-β is produced as a high molecular weight precursor molecule. The TGF-β1 precursor is cleaved by furin convertase to produce pro-TGF-β consisting of a mature TGF-β dimer non-covalently bound to their N-terminal ends (Dubois *et al.*, 1995). Latent TGF-β binding proteins are covalently linked to the propeptide portion of the molecule. These binding proteins are believed to be important for efficient extracellular secretion of the TGF-β complex (Miyazono *et al.*, 1991;Saharinen *et al.*, 1996). Activation of TGF-β can occur through a number of mechanisms. It has been shown that acidic and alkaline pH, heat, and urea treatment have the ability to activate latent TGF-β. Overall, it would appear that breaking of Hydrogen bonds is an important step in activation. At pH 3.0, latent TGF-β becomes fully activated (Brown *et al.*, 1990). The presence of acidic microenvironments in osteoclasts, activated macrophages and in the vicinity of solid tumours may be a causative factor *in vivo* in the activation of latent TGF-β (Silver *et al.*, 1988;Jullien *et al.*, 1989;Blair, 1998). Furthermore, TGF-β can be activated by proteolytic enzymes such as plasmin, cathepsin D, matrix metalloprotease-9 and calpain.

The glycoprotein Thrombospondin-1 and the integrin αvβ6 have also been shown to be involved in TGF-β activation (Lyons *et al.*, 1988;Schultz-Cherry and Murphy-Ullrich, 1993;Abe *et al.*, 1998;Munger *et al.*, 1999). Ultimately, TGF-β is only able to exert its potent effects when in its activated 25 kDa form.

1.1.2 TGF-β Receptors

The TGF-β ligands signal through a complex of two transmembrane serine/threonine kinases (Wrana *et al.*, 1992). Members of the TGF-β superfamily utilize different forms of Type I and Type II receptors (Fig 1) (Shi and Massague, 2003). Both TGF-β receptor I (TGF-βRI) and receptor II (TGF-βRII) contain an N-terminal extracellular ligand binding domain, a transmembrane domain, as well as a C-terminal kinase domain. TGF-βRI contains a SGSGSG motif preceding its kinase domain, termed the GS domain (Shi and Massague, 2003). The dimeric TGF-β ligand initially binds to the N-terminal ligand binding domain of the type II receptor before binding to the type I receptor. The type I receptor alone has no affinity for the ligand. The constitutively active TGF-βRII phosphorylates the type I GS domain, thereby activating the TGF-βRI kinase, producing an active receptor complex (Franzen *et al.*, 1995). Receptor complexes are continually internalized and recycled back to the plasma membrane in the absence of ligand while activated receptor complexes are sequestered by the cell and initiate downstream signaling (Dore, Jr. *et al.*, 2001). Receptor complexes are removed from the plasma membrane mainly via clathrin-mediated endocytosis (Anders *et al.*, 1997). It is believed that this endocytic pathway aids in the colocalization of active receptor

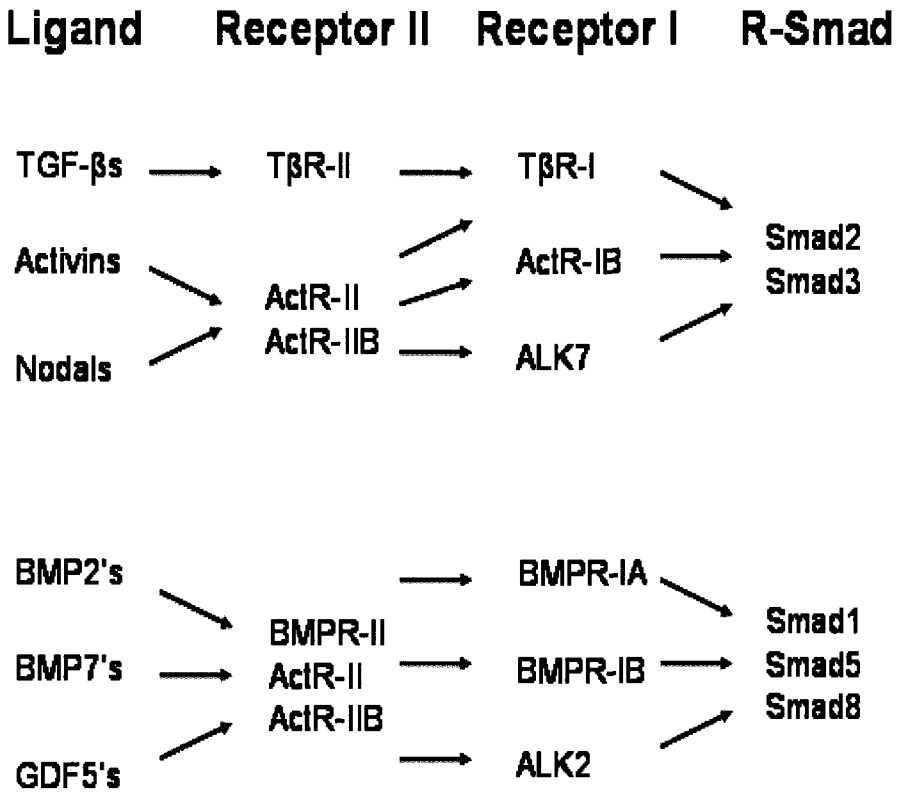

Fig 1. A schematic depiction of the Ligand-Receptor-R-Smad interactions for the members of the TGF-β superfamily of growth factors (Shi and Massague, 2003).

complexes with downstream components of the signaling pathway. TGF-βRs may also be internalized through the caveolar/lipid-raft-mediated pathway. Whereas clathrin-mediated endocytosis is thought to play a role in signal propagation, caveolar/lipid-raft-mediated endocytosis is integral for receptor degradation in a Smad7-Smurf2 dependant manner (Di Guglielmo *et al.*, 2003).

Within the TGF-β superfamily, the regulation of receptor activation is tightly controlled with both activators and inhibitors. Noggin and Chordin are secreted proteins that interact specifically with BMPs (Piccolo *et al.*, 1996;Zimmerman *et al.*, 1996). These proteins antagonize BMP signaling by interfering with the surfaces necessary for ligand interaction with type I and type II BMP receptors. Noggin regulates BMP function during vertebrate dorsal-ventral patterning, osteogenesis, and joint formation (Brunet *et al.*, 1998;Gong *et al.*, 1999). Chordin loss of function mutations in zebrafish produce a greatly reduced neural plate and an enlarged region of the ventral mesoderm (Hammerschmidt *et al.*, 1996). Follistatin is a glycoprotein that functions to suppress the release of follicle-stimulating hormone by binding to Activin and inhibiting receptor interaction (de Winter *et al.*, 1996). It has also been shown to interact with BMPs and effect signaling (Fainsod *et al.*, 1997).

TGF-β receptor activation can be controlled by a number of intracellular proteins including FKBP12 (Yao *et al.*, 2000). Through binding of TGF-BRI, FKBP12 prevents its phosphorylation via the type II receptor in a basal state, thereby preventing ligand-independent phosphorylation and activation of the receptor complex. It has also been shown to negatively regulate receptor internalization (Yao *et al.*, 2000). When TGF-β

ligand interacts with the type II receptor, FKBP12 is released from the receptor and its effect on regulation cease (Chen *et al.*, 1997).

1.1.3 Smads

Smad proteins are transcription factors specific to the TGF-β family of growth factors. In all, 8 Smad proteins are encoded in the human genome. Smads 1,2,3,5, and 8 act as downstream effectors for TGF-β family receptor complexes and are known as receptor-regulated Smads (R-Smads) (Macias-Silva *et al.*, 1996;Zhang *et al.*, 1996;Yamamoto *et al.*, 1997;Kretzschmar *et al.*, 1997b;Nishimura *et al.*, 1998;Nakayama *et al.*, 1998) (Fig 1). Smad4, also known as Co-Smad, is a common mediator for all R-Smads (Zhang *et al.*, 1997). Smads 6 and 7 function as inhibitory molecules, interfering with Smad-receptor and Smad-Smad interactions (Imamura *et al.*, 1997;Nakao *et al.*, 1997;Hayashi *et al.*, 1997;Hata *et al.*, 1998).

Smads are approximately 500 amino acids in length, made up of 3 distinct regions. The N-terminal, or "Mad Homology 1" (MH1) domain, is highly conserved in all Smad proteins, excluding Smads 6 and 7 (Massague, 1998). In an active state, both R-Smad and Co-Smad MH1 domains are involved in DNA-binding (Kim *et al.*, 1997;Liu *et al.*, 1997). In the basal state, the MH1 domain acts in an inhibitory fashion through its interaction with the C-terminal "Mad Homology 2" (MH2) domain (Hata *et al.*, 1997). The MH2 domain is also a highly conserved region of the protein. Contained within this region of R-Smads is a canonical Ser-X-Ser at the C-terminal end which is phosphorylated by the active receptor complex, leading to the activation of R-Smads

(Macias-Silva *et al.*, 1996;Kretzschmar *et al.*, 1997b). In addition, the MH2 domain mediates the interaction between R-Smads and the Type I receptor, Smad4, and DNA binding factors(Macias-Silva *et al.*, 1996;Hata *et al.*, 1997;Liu *et al.*, 1997). Separating these two conserved domains is a highly variable linker region. Among the R-Smads, this region contains several potential Mitogen Activated Protein Kinase (MAPK) sites and the phosphorylation of some of these sites is thought to contribute to the regulatory control of the molecule (Kretzschmar *et al.*, 1999).

Upon phosphorylation and internalization of the receptor complex, the type I receptor recruits R-smads for subsequent activation. This sequence of events requires the presence of an accessory protein called SARA (Smad Anchor for Receptor Activation). SARA contains a phospholipid binding FYVE domain which interacts with the membrane lipid PtdIns(3)P of early endosomes, thus allowing for more efficient recruitment of R-Smads to the active receptor complexes (Itoh *et al.*, 2002).

TGF-β stimulation results in the nuclear accumulation of R-Smads (Hoodless *et al.*, 1996;Souchelnytskyi *et al.*, 1997). Interestingly, it was shown recently that Smads are not confined to the cytoplasm in uninduced cells. R-Smad molecules are constantly shuttling between the cytoplasm and the nucleus. In the absence of TGF-β stimulation, the nuclear export rate is greater than the import rate, producing a greater cytoplasmic concentration of Smad proteins. When TGF-β is present, rate constants are shifted so that the nuclear export rate for the phosphorylated R-Smads is markedly decreased, increasing the phospho-smad concentration within the nucleus (Nicolas *et al.*, 2004;Schmierer and Hill, 2005).

Following activation, R-Smad-Smad4 oligomers are formed (Kawabata *et al.*, 1998). The makeup of these oligomers are still a matter of debate with recent evidence suggesting the formation of hetero/homo-dimers and hetero/homo-trimers of Smads 2 and/or 3 with the complex containing a single smad4 (Inman and Hill, 2002;Chacko *et al.*, 2004). Once in the nucleus, these Smad complexes are able to confer their regulatory properties on target genes. The promoter regions of these Smad-responsive genes contain sites called Smad Binding Elements (SBE) (Zawel *et al.*, 1998). These sites are recognized by a β-hairpin in the Smad MH1 domain (Shi *et al.*, 1998). The affinity of Smad proteins for a single SBE is too low for efficient binding of the Smad complex to the promoter region of the target gene. However, even promoters containing multiple SBEs rely on co-factors to aid in the binding ability of Smad complexes (Seoane *et al.*, 2004). These co-factors function to increase transcription and are known as coactivators. Some coactivators include CBP/p300, ARC105, and Swift (Janknecht *et al.*, 1998;Pouponnot *et al.*, 1998;Shimizu *et al.*, 2001;Kato *et al.*, 2002). These molecules increase transcription by interacting with the Smad complexes and recruiting them to the RNA polymerase II complex. Additionally, some coactivators like CBP and p300 have histone acetyltransferase activity to modify chromatin structure, allowing for Smad regulation of transcription (Ross *et al.*, 2006). Alternatively, certain co-factors aid in repressing transcription via Smads. These corepressors include c-Ski/SnoN and TGIF (Akiyoshi *et al.*, 1999;Stroschein *et al.*, 1999;Wotton *et al.*, 1999;Xu *et al.*, 2000). These molecules interact with the MH2 domain of Smad2 and Smad3. TGIF has the ability to directly interact with histone deacetylases (HDACs) whereas c-Ski and SnoN interact

with the nuclear transcriptional corepressor (NCoR) that recruits HDACs (Wotton *et al.*, 1999;Luo *et al.*, 1999).

Termination signaling appears to occur in a number ways. Lin et al, (Lin *et al.*, 2006) demonstrated that within epithelial cells, the phosphatase PPM1A dephosphorylates R-Smads terminating TGF-β signaling. Additionally, Smad2 is ubiquitinated in the nucleus and undergoes degradation via the 26S Proteasome (Lo and Massague, 1999). Termination of signaling also occurs at the receptor level where the ubiquitin ligases Smurf1 and Smurf2 (Smad ubiquitin regulatory factor) mediate ubiquitination of activated TGF-β receptors. Both Smurf1 and Smurf2 form a complex with Smad7. This complex is then localized to the plasma membrane where Smad7 binds to the type I receptor, inhibiting R-Smad phosphorylation and promoting receptor turnover (Kavsak *et al.*, 2000;Ebisawa *et al.*, 2001). Smad6 antagonizes BMP signaling by competing with Smad1 in binding to Smad4 (Hata *et al.*, 1998). Through the regulation of receptor activation and internalization, R-Smad dephosphorylation and degradation, complex formation of Smads and their co-activators/repressors, this subset of Smads represents another method by which TGF-β signaling is controlled.

1.1.4 TGF-β/Smad Mutations in Cancer

TGF-β is a potent regulator of a number of cellular processes such as proliferation, migration, cell survival and angiogenesis. The manner in which TGF-β regulates growth is cell type specific; TGF-β promotes growth in mesenchymal cells and inhibits growth in epithelial, endothelial and hematopoietic cells. Mutation or deletion of

aspects within the TGF-β signaling pathway are frequently seen in human cancers. Approximately 85% of all tumours are epithelial in origin, some of which lose their sensitivity to the growth inhibitory effects of TGF-β (Elliott and Blobe, 2005). In some types of cancer, the inherent resistance can be associated with defects in the Smad proteins. In pancreatic cancer for example, Smad4 was originally referred to as *deleted in pancreatic cancer 4* [DPC4], is inactivated in approximately 50% of all pancreatic adenocarcinomas (Hahn *et al.*, 1996) and mutated in one third of metastatic colon cancers (Miyaki *et al.*, 1999). Mutations leading to the inactivation of Smad2 are primarily found in colon and lung cancers (Eppert *et al.*, 1996;Uchida *et al.*, 1996), while no mutations of the Smad3 gene have been reported in studied carcinomas (Derynck *et al.*, 2001). However, in the Smad3 knockout mouse model, adult mice have a high occurence of colon cancer, suggesting Smad3 may be a risk factor (Zhu *et al.*, 1998).

TGF-β receptors are also subject to defects and play a role in the proliferation of human cancers. Nucleotide additions or deletions of the gene encoding TGFβRII are often found in patients with hereditary non-polyposis colorectal cancer (HNPCC) (Derynck *et al.*, 2001). Mutations which lead to the inactivation of TGFβII have been found in approximately 20-25% of colon cancer patients (Derynck *et al.*, 2001). Furthermore, mutated forms of TGFβR2 have also been found in gastric, prostate, and breast cancers, as well as gliomas (Elliott and Blobe, 2005). TGFβR1 inactivating mutations have also been seen in ovarian cancers, metastatic breast cancers, pancreatic carcinomas and T-cell lymphomas (Chen *et al.*, 1998;Goggins *et al.*, 1998;Schiemann *et al.*, 1999;Chen *et al.*, 2001)

Smad Independent Signaling

1.2.1 Ras Signaling

Ras proteins are members of a large superfamily of small G-proteins with GTPase activity (Paduch *et al.*, 2001). Other members in this superfamily include the Rho family, that play a role in actin cytoskeleton regulation, as well as the Rab family, that regulate intracellular vesicular trafficking (Tapon and Hall, 1997;Novick and Zerial, 1997). GTPases function as molecular switches by binding GTP or GDP. In their inactive form, GDP is bound. To activate the GTPase, the bound GDP is replaced by GTP. This exchange is moderated by Guanine Nucleotide Exchange Factors (GEFs). GEFs work by promoting the dissociation of bound GDP and the uptake of GTP from cytosol stores (Bos *et al.*, 2007). Conversely, GTPase-Activating Proteins (GAPs) promote the hydrolysis of bound GTP, subsequently inactivating the G-protein (Bos *et al.*, 2007).

The upstream signals that result in the activation of Ras have been linked to the tyrosine kinase receptors (Kamata and Feramisco, 1984). This signaling often leads to cellular proliferation or differentiation. Thus, the presence of a mutation constitutively activating Ras can lead to uncontrolled cellular growth. Approximately 30% of human tumours contain activated Ras mutations (Bos, 1989;Campbell and Der, 2004). One of the ways in which Ras propagates proliferative signals is through the MAPK signaling cascade (Robbins *et al.*, 1992). Acting directly downstream of Ras is a family of serine/threonine MAP3Ks, composed of Raf-A, -B, and -C. Ras phosphorylates Raf which in turn, phosphorylates the MAPK2K MEK1/2. This subsequently activates the MAPK Erk (Roberts and Der, 2007).

1.2.2 PI3K/AKT Pathway

Upstream of Ras is the PI3K/Akt signaling pathway, a major component of growth factor receptor signaling, regulating cellular functions such as proliferation and cell survival (Brazil *et al.*, 2004). There are 3 classes of PI3Ks with the Class I being primarily associated with Receptor Tyrosine Kinase (RTK) signaling although activation of PI3K can be initiated by tyrosine kinase and serine/threonine receptors (Hadari *et al.*, 1992;Bakin *et al.*, 2000;Brazil *et al.*, 2004). PI3K Class I functions by adding a phosphate group to the membrane phosphatidylinositol PI(4,5)P_2, converting it to phosphatidylinositol (3,4,5)-triphosphate (PIP_3) (Stephens *et al.*, 1993;Hawkins *et al.*, 2006). The PI3K phospholipids products PIP2 and PIP3 attracts proteins containing Pleckstrin homology (PH) domains which have a strong affinity for the 3' phosphorylated inositol head group. One such protein is a serine/threonine kinase, protein kinase B (PKB), also known as Akt. Akt recruitment to the plasma membrane by phosphoinositides is necessary for its activation (Franke *et al.*, 1997;Frech *et al.*, 1997). Akt imparts a multitude of effects on the cell through its phosphorylation of many proteins. Akt promotes proliferation through its inhibition of GSK-3β (Cross *et al.*, 1995) and FOXO4 (Kops *et al.*, 2002). It prevents apoptosis by inhibiting Bad, caspase-9, and FOXO1, and by activating anti-apoptotic proteins such as Mdm2 (Cross *et al.*, 1995;Datta *et al.*, 1997;Cardone *et al.*, 1998;Brunet *et al.*, 1999;Mayo and Donner, 2001). As important nodes of cellular controls, both PI3K and Akt activation must be tightly regulated. This is done through lack of growth stimulation in quiescent cells, or through phosphatases, such as PTEN, that antagonize the actions of PI3K (Gao *et al.*, 2000).

Mutations leading to constitutive activition of PI3K or inactivation of PTEN result in deregulation of the signaling pathway. A number of these types of mutations have been characterized in a number of cancer types including Glioblastoma, breast and ovarian carcinoma, as well as prostate and colon carcinoma (Vivanco and Sawyers, 2002).

1.2.3 Ubiquitin Dependent Protein Degradation

Regulation of many cellular proteins is maintained through the ubiquitin-proteasome system. This system selectively regulates certain protein concentrations depending on physiologic signals and cellular conditions. In addition to being specific, this form of protein regulation also has the advantage of being unidirectional, rapid, and localized to specific cellular compartments. In many cases, proteins are marked for degradation through phosphorylation by kinases. This phosphorylation allows for the covalent attachment of multiple ubiquitin molecules, a process known as polyubiquitylation. Ubiquitin ligase, a complex made up of three proteins (E1, E2, and E3), initially recognize a protein for degradation and then flags the protein with ubiquitin chains (Ciechanover, 1998). Once ubiquitinated, the tagged protein is localized to a proteasome. The proteasome is large, hollow and comprised of multiple protein subunits. Hydrolysis of ATP catalyzes the unfolding of the protein, which is then de-ubiquitinated and digested into short peptide fragments (Ciechanover, 1998).

1.2.4 Smad Independent Pathways

Recently, a number of other proteins have shown to be affected upon TGF-β receptor activation such as PI3K. Studies have shown that PI3K is activated in a cell type specific manner. The initiation of PI3K activity in mesenchymal cells, but not epithelial cells leads to the activation of p21-activated kinase 2 (PAK2) (Wilkes *et al.*, 2005). PI3K activation can be directly initiated RhoA or indirectly through TGF-β induced TGF-α expression. PAK2 has normally been shown to be involved in cytoskeletal reorganization while its budding yeast homologue, Ste20, has the ability to act as a MAP4K in cellular signaling (Dan *et al.*, 2001). RhoA and its effector kinase $p160^{ROCK}$, in addition to the p38 pathway, are believed to be important in Smad-dependent growth inhibition of breast carcinoma cells (Kamaraju and Roberts, 2005). Furthermore, the protein phosphatase PP1 interacts with the Drosophilia SARA. This interaction disrupts type I receptor phosphorylation, negatively regulating TGF-β signaling (Bennett and Alphey, 2002). In all cases however, the mechanisms by which activation occurs, or their biological relevance in terms of TGF-β signaling are poorly understood.

1.2.5 TGF-β/MAPK pathway

In addition to mediating Smad induced transcription, TGF-β family members have also been shown to initiate a number of independent signaling cascades including members of the MAPK family. MAPKs are categorized into three subfamilies; the extracellular signal regulated proteins (Erk1/2), stress activated proteins c-Jun N-terminal kinase (JNK1, JNK2, JNK3) and p38/MAPKs (Chang *et al.,* 2001). One study

demonstrated that a member of the MAPKKK family, TGF-β Activated Kinase1 (TAK1), was stimulated in response to TGF-β and BMP signals (Yamaguchi *et al.*, 1995). In over-expression studies, TAK1 has been demonstrated to act upstream of the MAPKs JNK, and p38. TAK1 activates p38 and JNK via MKK3/6 and MKK4/7 respectively (Moriguchi *et al.*, 1996;Wang *et al.*, 1997;Bakin *et al.*, 2002). Furthermore, extracellular signal regulated kinase1/2 (Erk1/2) can also be activated through TGF-β signaling (Hayashida *et al.*, 2003). TGF-β activation of MAPKs can be cell-type specific. As an example, in rat articular chondrocytes, TGF-β activates ERK1/2 but neither JNK or p38 (Yonekura *et al.,* 1999). The activation of MAPKs via TGF-β has been shown to be smad-independent through experiments utilizing mutant Type I receptors which are unable to phosphorylate R-smads, that are still able to activate p38 after TGF-β stimulation (Yu *et al.,* 2002). The mechanisms by which any of the MAPK members are activated through TGF-β remain poorly understood.

1.2.6 Crosstalk Mechanisms

A central caveat in TGF-β signaling is the ability of the Smad-independent pathways to interact and regulate Smad proteins. Up until recently, there has been no definitive evidence showing which kinases are involved or how they act on Smads. Much focus has been placed on the highly variable Smad linker regions. R-Smad linker regions contain potential phosphorylation sites for a number of kinases including Erk, p38, Rho/ROCK, cyclin-dependent kinases (CDKs), c-Jun N-terminal kinase and Ca^{2+}/calmodulin-dependent kinase II (Yue *et al.*, 1999;Wicks *et al.*, 2000;Matsuura *et al.*,

2004;Mori *et al.*, 2004;Kamaraju and Roberts, 2005). Phosphorylation of 4 sites within the linker region of Smad1 was shown to occur by Epidermal Growth Factor (EGF) induced Ras/MAPK activation and appeared to inhibit both nuclear localization and BMP signaling (Kretzschmar *et al.*, 1997a). Furthermore, mutation of three potential MAPK sites within the linker region of *Xenopus* Smad2 also resulted in inhibition of nuclear translocation (Grimm and Gurdon, 2002). Alternatively, other studies have noted that Erk phosphorylation positively regulates Smad transcriptional activity and does not inhibit nuclear translocation (Engel *et al.*, 1999;Funaba *et al.*, 2002). The interaction between TGF-β induced pathways may have a negative effect. Smad6 can downregulate TAK1 activity (Kimura *et al.*, 2000). Through JNK signaling, c-Jun inhibits Smad2 signaling through association with Smad co-repressors (Pessah *et al.*, 2002). Furthermore, interaction between TGF-β induced Smad and MAPK signaling is important in epithelial-to-mesenchymal (EMT) transition, as both Erk and p38 play a role in EMT (Bakin *et al.*, 2002;Davies *et al.*, 2005). It is clear that much remains to be learned regarding the interactions between Smad-dependent and Smad-independent pathways before a clear and concise answer can be found.

1.3.1 Thesis Rationale and Hypothesis

TGF-β serves as a potent regulator of a variety of biological effects. TGF-β has been shown to regulate the actions of a number of downstream molecules. The Smad signaling cascade has been studied extensively. Smad-independent pathways however, are poorly understood. The objective of this study was to define the role of TGF-β in Erk

activation by characterizing the mechanisms of the pathway by which Erk phosphorylation is initiated. We also sought to determine the ability of Erk to interact with the Smad signaling pathway, as well as to define a functional role for Erk in TGF-β signaling. We believe that TGF-β initiates Erk activation by mechanisms distinct from the classical MAPK pathway, possibly through PI3K and PAK2. As Erk is involved in cellular proliferation and differentiation, its subsequent activation would result in a growth stimulatory affect through its positive regulation of certain transcription factors and negative regulation of the canonical TGF-β induced smad pathway.

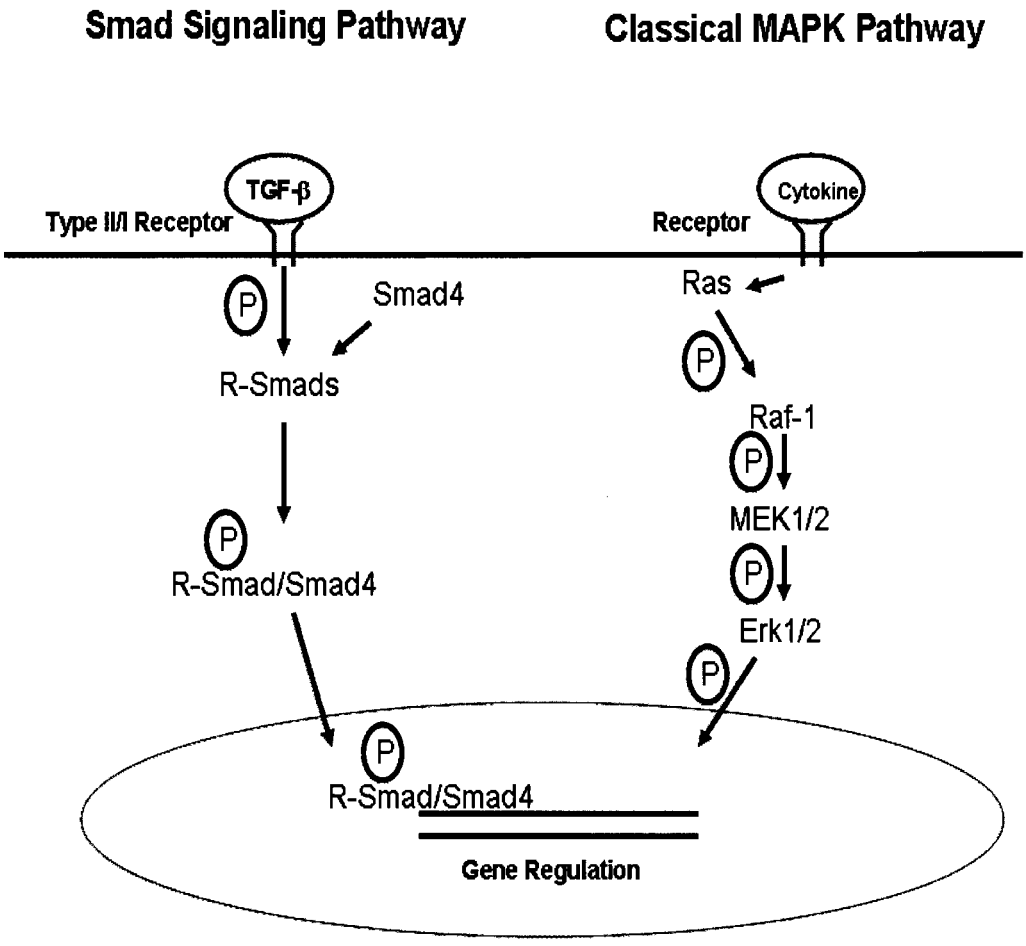

Fig 2. **Canonical Smad signaling pathway and the classical MAPK pathway.** A depiction of the mechanisms involved in smad and MAPK signaling.

CHAPTER 2 - Introduction

Transforming Growth Factor β (TGF-β) is a constituent of a family of structurally related cytokines that control a myriad of cellular functions. TGF-β elicits its cellular responses by signaling through a receptor complex of serine/threonine kinase type I (TβRI) and type II (TβRII) receptors at the cell surface (Lin *et al.*, 1992;Franzen *et al.*, 1993). Ligand binding to the receptor complex requires clathrin mediated endocytosis prior to signaling through recruitment of receptor mediated (R-) Smad2 and/or Smad3. Phosphorylation at the putative C-terminal SSXS motif on Smad2/3 allows them to complex with the common mediator (Co-) Smad4 (Zhang *et al.*, 1996;Zhang *et al.*, 1997). The Smad complex then translocates into the nucleus, resulting in the regulation of target gene expression (Macias-Silva *et al.*, 1996;Baker and Harland, 1996). Interestingly, although both mesenchymal and epithelial cells contain the canonical TGF-β/Smad signaling cascade, epithelial cell types are growth inhibited, whereas mesenchymal cells are growth stimulated by TGF-β. This would suggest a fundamental mechanistic difference in TGF-β signaling between cell types, independent of the Smad signaling cascade.

In addition to Smad signaling, TGF-β has also been implicated in the initiation of a number of Smad-independent pathways including Erk, Jnk, ROCK, and more recently, PI3K/PAK2. The p21-activated kinases (PAK1-6) are the mammalian homologues of the Ste20 group of kinases, originally defined in yeast as part of the Ras signaling pathway (Dan *et al.,* 2001). In mammals PAKs have been found to be regulated by PI3K through cdc42/Rac1 (Wilkes *et al.*, 2003). Recently, PAK2 has been shown to be activated

specifically in mesenchymal cells, downstream of TGF-β receptor and PI3K activation and may be associated with TGF-β activation of mammalian Ras signaling (Wilkes *et al.*, 2003;Suzuki *et al.*, 2007). Functionally, PAKs are Serine/Threonine kinases that participate in apoptosis, cell motility and cytoskeletal rearrangement (Hofmann *et al.*, 2004). Of note are recent findings suggesting Ste20 kinases upstream of mitogen activated protein kinase (MAPK) signaling cascades, implicating them as potential MAP kinase kinase kinase kinases (MAP4K) (Dan *et al.*, 2001). This raises an interesting possibility that the Ste20 mammalian homologue, PAK, has the potential to act in a similar fashion.

The coupling of the MAPK signaling cascade with the Smad pathway has long been suggested in TGF-β signaling (Kretzschmar *et al.*, 1997a;Kretzschmar *et al.*, 1999), but the relationship and mechanism this occurs by is still unknown. Within the linker region, and to a lesser extent the MH1 and MH2 domains of Smad2 and Smad3, are potential MAPK phosphorylation sites. Extracellular Signal Regulated Kinase1/2 (Erk) phosphorylation of sites within the linker region have been shown to both inhibit Smad nuclear translocation and enhance Smad mediated transcriptional activity, two mutually exclusive functions (Yue *et al.*, 1999;Kretzschmar *et al.*, 1999;Hayashida *et al.*, 2003). The classic Erk cascade, typically starts with receptor tyrosine kinases such as Epidermal Growth Factor (EGF), that activate the small G protein Ras or as in some cancers by mutations rendering Ras constitutively active (Kretzschmar *et al.*, 1999;Lo *et al.*, 2001). Active Ras then recruits and activates one of the MAP kinase kinase kinases (MAP3K) in the Raf family, that subsequently phosphorylate the MAP2K, Mek1/2, that in turn

activate Erk1/2 through phosphorylation. Once activated in the cytoplasm, Erks translocates into the nucleus and effects factors responsible for cell cycle progression such as cyclin D1 and transcription factors such as Elk-1 and c-Myc (Gille *et al.*, 1995;Weber *et al.*, 1997;Sears *et al.*, 1999;Sears *et al.*, 2000) .

In addition to their roles in cytoskeletal regulation, PAKs may play a role in regulating the activity of both c-Raf and Mek1 (Coles and Shaw, 2002;Park *et al.*, 2007). A recent study reported a mechanistic difference between EGF and platelet-derived growth factor (PDGF) within their respective pathways leading to the activation of Erk (Beeser *et al.*, 2005). Inhibition of all three Group A PAKs (PAK1,2,3) led to a decrease in Erk activation in response to PDGF, but not EGF. To date, there have been no specific studies aimed at delineating the mechanisms of, or the conditions for TGF-β induced Erk activation.

Erk is an effector of Ras signaling. As such, constitutively active forms of Ras drive persistent Erk activation. Approximately 30% of all human cancers contain mutant forms of Ras (Malumbres and Barbacid, 2003). In addition to Erk, Ras has been known to use other mediators to affect tumourigenesis. The class I PI3K molecules are known interactors with Ras (Roberts and Der, 2007). TGF-β initiates class I PI3K activation (Bakin *et al.*, 2000). In addition to activating Akt, PI3K activates the small GTPases Rac1 and cdc42, which then activate PAK2 (Wilkes *et al.*, 2003). The PI3K/Akt pathway is integral to the TGF-β induced epithelial-to-mesenchymal transition (Bakin *et al.*, 2000;Bhowmick *et al.*, 2001). PI3K has also been shown to be an integral part of receptor tyrosine kinase signaling through the Ras family of GTPases (Shaw and Cantley, 2006).

The Ras family of small GTPases have been shown to play an integral role TGF-β induced signaling. Activation of both Rac and cdc42 have been shown to regulate JNK and p38 affecting cytoskeletal organization (Bishop and Hall, 2000). The activation of Rac1, cdc42 and PAK2 by TGF-β has been identified as a cell type specific affect. Rac1 and RhoA activation has also been shown to be necessary for TGF-β induced membrane ruffling and lamellipodia formation (Edlund *et al.*, 2002). The data presented in this thesis suggests an interaction between smads and PI3K-PAK2-Erk in the regulation of fibroblast growth. This may be a mechanism that TGF-β signaling utilizes to control smad signaling.

In order to maintain balance and prevent signaling systems from overexerting their affects, controls must be in place within the system. One level of control of TGF-β induced signaling is via Ubiquitin-proteasome-mediated degradation. Smurf1 and Smurf2 are members of the HECT family of E3 ubiquitin ligases, with Smurf1 affecting BMP signaling through interaction with Smad1 and Smad5 (Zhu *et al.*, 1999), and Smurf2 affecting both TGF-β and BMP related R-Smads (Zhu *et al.*, 1999;Arora and Warrior, 2001;Bonni *et al.*, 2001). Smad2 is thought to be regulated by the proteasome since inhibition of Smad2 degradation increases its nuclear accumulation (Lo and Massague, 1999). C-terminally phosphorylated Smad3 interacts with the protein Roc1, promoting an interaction with an SCF ubiquitin ligase complex and subsequent nuclear exclusion and cytoplasmic proteasomal degradation (Fukuchi *et al.*, 2001). Unlike the R-Smads, Smad4 does not appear to be under the control of the ubiquitin-proteasome system.

Within this study, we define a novel mechanism for cell type specific TGF-β activation of Erk. We show only in mesenchymal cell lines was TGF-β able to induce Erk activation through activation of PAK2 via PI3K. Additionally, Ras does not appear to be involved in TGF-β induced phosphorylation of Erk, but may in fact negatively impact Erk activation. Furthermore, the expression of an unknown protein is necessary for TGF-β induced Erk activation, as inhibition of either RNA or protein synthesis abolished Erk phosphorylation. Moreover, the regulation of nuclear Smad2 in fibroblasts appears to be under the control of the ubiquitin-proteasome system. In the presence of TGF-β stimulation, nuclear Smad2 increases upon inhibition of the proteasome (Lo and Massague, 1999). As such, the lag of Erk activation relative to the quick activation of the Smad signaling system may be a cellular method to constrain the long-term growth inhibitory affects of Smads and allow for the promotion of TGF-β induced growth normally seen in fibroblasts. We also show that activated Erk is integral to the growth stimulatory effect of TGF-β within fibroblasts potentially through its interactions with the transcription factor c-myc, in addition to Smad2. Erk appears to phosphorylate Smad2 within the linker region only after both translocate to the nucleus. Taken together, this study defines a PI3K/PAK2/Erk pathway utilized by TGF-β, demonstrating the requirement of this pathway in mesenchymal growth regulation, a direct link between TGF-β signaling and the regulation of the proto-oncogene myc and an interaction between this pathway and the classical Smad pathway.

CHAPTER 3 - Materials and Methods

3.1 Cell Culture

All cell lines used were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM; GIBCO). Fibroblast cell lines (AKR-2B) were grown in DMEM supplemented with 5 % (v/v) Fetal Calf Serum (FCS), while NIH-3T3 cells were grown in DMEM supplemented with 10% (v/v) Newborn Calf Serum (NBCS). Epithelial cell lines (Mv1Lu, HEK293) were grown in DMEM supplemented with 10% (v/v) FCS, while NMuMG cell growth media also contained 10μg/ml bovine Insulin (Sigma) and 5ng/ml EGF. Cells were grown to approximately 80% confluency prior to each passage. Cells were maintained for approximately 18-20 serial passages before being discarded and new stocks thawed from liquid nitrogen. All cells were maintained at 37°C in an atmosphere of 5% CO_2/air.

3.2 Western Blotting

Fibroblast cell lines were removed from T175 flasks by trypsinization and resuspended in normal growth media. Into each well of a 6 well plate, $3x10^5$ cells were plated at a concentration of $1.5x10^5$ cells/ml. After allowing cells to attach for 24 hours, media was changed to 0.1% NBCS to serum deprive cells for 18 hours prior to experimentation. Epithelial cell lines were plated at a concentration of $1.0x10^5$ for NMuMG cells and $3.0x10^5$ cells/ml for Mv1Lu cells. After being allowed to attach for 24 hours, NMuMG cells were growth factor deprived by replacing the growth medium with

10% FCS/DMEM for 18 hours before treatment. Mv1Lu cells were treated approximately 18 hours after plating. Cultures were then stimulated with TGF-β at a concentration of 2ng/ml for indicated time points. To inhibit PI3K and MEK1/2 activity, LY294002 (Upstate) and U0126 (Cell Signal Technologies) respectively were used at concentrations of 10μM dissolved in DMSO. To obtain total cellular protein samples, cells were lysed in RIPA (1X PBS, 1X complete protease inhibitor [Roche], 1% Triton X-100, 50mM Tris-HCl [pH 7.4; EM Science], 50 mM β-Glycerophosphate [Sigma], 50mM Sodium Fluoride [EM Science], 0.1% SDS, 0.1 μM Sodium Orthovanadate, 5mM EDTA [EM Science], 75ng/μl PMSF [Roche]). Cellular lysate samples were quantified for total protein using a BCA assay with a standard curve generated with a BSA standard (Pierce Biotechnologies). Aliquots containing equivalent total protein from each sample were then separated in an 8.5% polyacrylamide gel, transferred to PVDF (Millipore) or Nitrocellulose (Bio Rad) membrane prior to antibody detection of each specific protein. Membranes were blocked in Blotto Buffer (5% w/v Nonfat Dry Milk in TBST [10mM Tris-HCl pH 7.4, 150mM NaCl, 0.1% v/v Tween-20]) for PVDF or 5% BSA/TBST for Nitrocellulose for 1 hour and then incubated overnight at 4°C in primary antibody. All antibodies used were from Cell Signal Technologies and included p44/22 MAP Kinase (#9102), Phospho-p44/22 (Thr202/Tyr204; #9101), Phospho-AKT (Ser473; #9427), and AKT (#9272), Phospho-Smad2 (Ser245/250/255; #3104), Phospho-Smad2 (Ser465/467; #3101), Smad2 (3102), Phospho-c-Raf (Ser338; #9427), PAK2 (#2608), and Phospho-c-myc (Thr58/Ser62; #9401). Membranes were washed 5 times for 5 minutes each in TBST and incubated for 1 hour at room temperature in Goat anti-Rabbit IgG-HRP secondary

antibody (Santa Cruz) in either Blotto Buffer (PVDF) of 5% BSA/TBS (Nitrocellulose) at a 1:15 000 dilution. Membranes were then washed 5 times at 10 minutes per wash in TBST before addition of Supersignal West Pico Chemiluminscent Substrate (Pierce) for 5 minutes and then exposed to film (Hyperfilm, GE Health).

3.3 Immunocytochemistry

NIH 3T3 cells were plated at a concentration of 20 000 cells/well on a 4 chamber slide (Labtech) and allowed to attach for 24 hours. Cells were then serum deprived for 18 hours prior to being treated with TGF-β (2ng/ml) for 3 hours. To terminate signaling, the cells were washed with ice cold PBS and fixed with 4% Paraformaldehyde/PBS for 30 minutes at 4°C. Cells were washed for 5 minutes with TBS and permeabilized using 0.2% Triton X-100 for 2 minutes at room temperature. Cells were then washed with 3 changes of TBST prior to blocking. Blocking solution (PBS, 5% BSA [Sigma], 10% normal goat serum [Sigma]) was added to each chamber and cells were incubated at 4°C for 60 minutes. Phospho-Smad2 (Ser 245/250/255) antibody was diluted 1:100 in blocking solution and filtered through a 0.20μm filter before addition to each chamber and incubated overnight at 4°C in a humidified chamber. The cells were then washed 3 times in TBST for 5 minutes and incubated in Rhodamine X conjugated secondary antibody (1:300 dilution in blocking solution; Jackson labs) at room temperature for 30 minutes in the dark. Cells were then washed 3 times for 5 minutes in TBST under low light conditions before being coverslipped using Vectashield mounting media (Vectorlabs).

3.4 Cellular Fractionation

Nuclear and cytoplasmic protein fractions from TGF-β treated AKR-2B cells were prepared using the NE-PER Nuclear and Cytoplasmic Fractionation kit as described by the manufacturer (Pierce Biotechnologies). Cells were plated in 6 well tissue culture plates at $3x10^5$ cells/well and allowed to attach for 24 hours. Growth medium was exchanged for 0.1% NBCS/DMEM, to serum deprive cells for 18 hours prior to being treated with TGF-β (2ng/ml) for the indicated time points. Cells were washed with ice cold PBS and scraped from the wells using a cell scraper into 200μl PBS. Cells from 3 wells were combined and pelleted at 500 x g for 5 minutes and the supernatant removed. CERI reagent was added to each cell pellet and cells were resuspended by vortexing for 15 seconds followed by incubation on ice for 10 minutes. CERII reagent was added to each sample before vortexing, incubated on ice for 1 minute, vortexed briefly and centrifuged for 5 minutes at 15 000 x g. The supernatant (cytoplasmic extract) was then transferred to a new microcentrifuge tube. The pellet was treated with NER reagent and vortexed for 15 seconds every 10 minutes for a period of 40 minutes. The samples were centrifuged for 10 minutes at 15 000 x g and the supernatant (nuclear extract) transferred to a new tube and both fractions were placed at -80°C for storage.

3.5 Thymidine Incorporation

The assay carried out was based on previously described methods (Wharton *et al.*, 1982). NIH 3T3 cells were plated at 40 000 cells/well in a 24 well plate and allowed to attach for 24 hours. Cells were then serum deprived in 0.1% NBCS for 24 hours and then

treated with TGF-β (5 ng/ml), EGF (20ng/ml), and/or U0126 (10μM) for 18 hours. 1μCi Tritiated Thymidine (Amersham) was added to each well and incubated for 2 hours. Media was then aspirated and incorporated radioactivity was precipitated with 2 washes of 10 minutes with 1ml ice cold 10% Trichloroacetic Acid (TCA). The TCA was then aspirated and 300μl solublization buffer (0.2N NaOH, 200 μg/ml ssDNA) was added to each well and shaken at room temperature for 30 minutes. Incorporated radioactivity was quantified by adding 100μl from each sample to 5ml scintillation fluid and counted using a Beckman Coulter Ls6500 Liquid Scintillation Counter.

3.6 Adenovirus Infection

Dominant negative PAK2-EGFP fusion protein and EGFP-expressing adenoviruses was generously provided by Dr. Ed Leof from the Mayo Clinic, Rochester, Minnesota. AKR-2B cells were plated at a concentration of 1.5×10^5 cells/ml in DMEM containing 10% FCS, and incubated for 8 hours before addition of the virus. For adenoviral infection, an MOI of 125:1 was used. Approximately 18 hours after addition of virus, medium was replaced with DMEM containing 0.1% Newborn Calf Serum (NBCS). After another 18 hours, cells were treated with TGF-β (2ng/ml) for 3 hours and then total cellular lysate was prepared as previously described.

CHAPTER 4 - Results

4.1 TGF-β Activates Erk in a Cell Type Specific Manner

As a starting point, we decided to test multiple mesenchymal and epithelial cell lines to determine if there was any variation in Erk activation. Levels of Erk phosphorylation were determined in AKR-2B fibroblasts treated with 2 ng/ml TGF-β2 over the course of 3 hours. As shown in Fig. 3A, Erk phosphorylation begins to appear approximately 60-90 min. after TGF-β addition. Similar results were obtained in NIH 3T3 fibroblasts (Fig. 3A), indicating Erk activation is not cell line specific, but a general property of fibroblasts. Other investigators have shown Erk activation at earlier time points. We likewise saw an increase at 30 minutes, but only when cells were allowed to cool down during addition of TGF-β. If cells were maintained at near 37°C, no activation of Erk was seen prior to the 60-90 minute window. To further this concept, the experiment was repeated using Mv1Lu and NMuMG epithelial cells where no increase in Erk phosphorylation was seen at any point (Fig. 3B). As a control, NMuMG cells were also treated with 50 ng/ml EGF to demonstrate that Erk and the MAPK signaling cascade functions in these epithelial cell lines. Together these results suggest that the activation of Erk upon TGF-β treatment occurs in cells of mesenchymal origin, but not in epithelial cells.

4.2 PI3K/PAK2 Function is Necessary for Activation of Erk

Having established Erk activation as a cell type specific event, the next step was to determine the upstream components through which this signal is propagated.

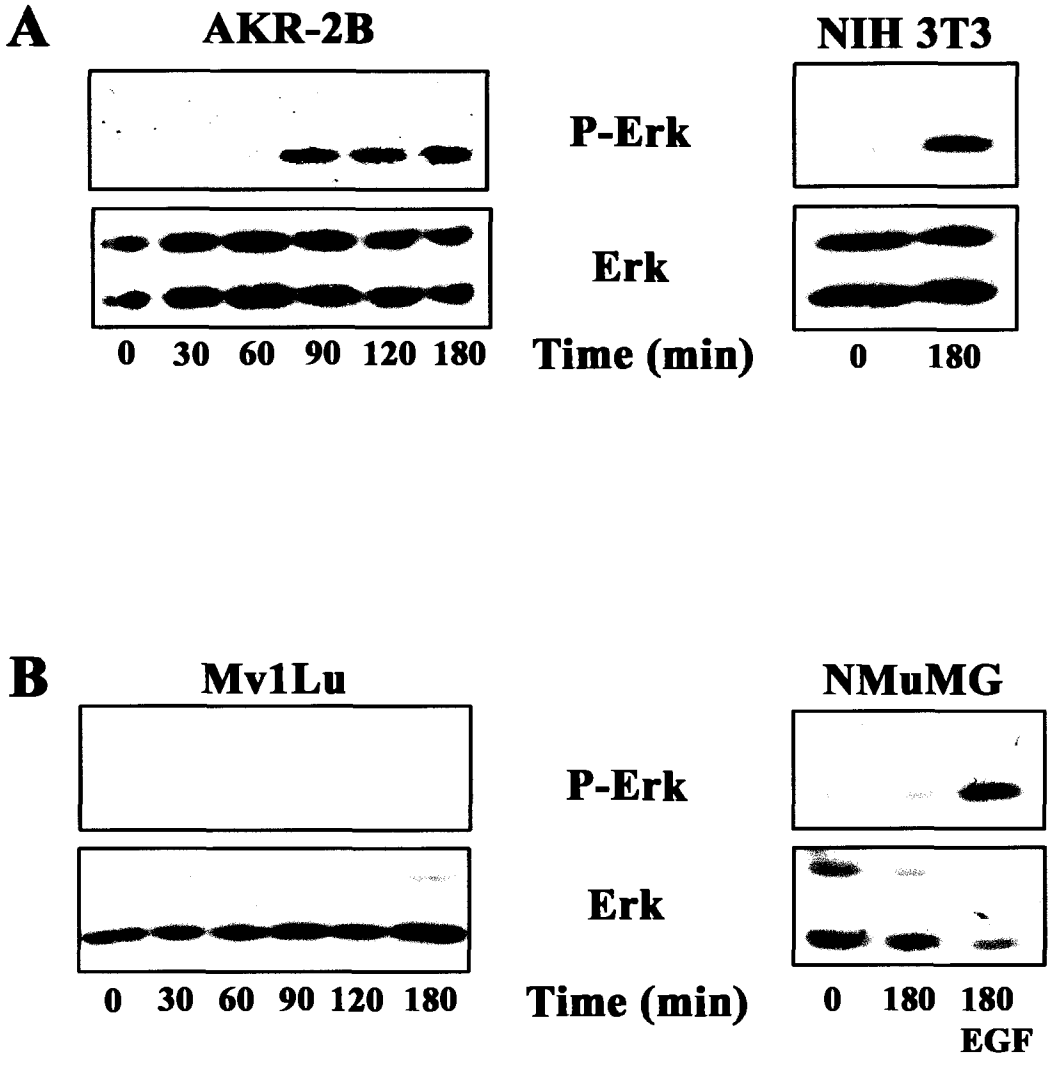

Fig. 3. **Cell type specific activation of Erk. (A)** AKR-2B and NIH 3T3 fibroblast, as well as **(B)** Mv1Lu and NMuMG epithelial cell lines were treated with TGF-β (2ng/ml) for times ranging from 0 to 3 hours. EGF (50ng/ml) was used as a positive control in epithelial cells. Cell lysates were probed with antibodies specific to phospho-Erk (P-Erk). Blots were then stripped and reprobed for total Erk as a loading control. Assays were performed in triplicate.

Previous studies have shown that TGF-β induced activation of PI3K/PAK2 is specific to fibroblasts (Wilkes *et al.*, 2003;Wilkes *et al.*, 2005). As our results are indicative of the same trend, we sought to discover whether or not Erk is in fact downstream of this pathway. We first determined the relationship between PI3K and Erk using LY294002, a specific inhibitor of PI3K function. Inhibiting PI3K produced a substantial decrease in Erk phosphorylation following treatment with TGF-β (Fig. 4A), indicating that PI3K is involved in TGF-β activation of Erk.

In the classic MAPK signaling pathway as described for tyrosine kinase receptors, Erk phosphorylation follows the activation of MEK, which is activated by c-Raf. Since it is unknown how TGF-β may be activating Erk, we wanted to determine if TGF-β utilizes similar signaling intermediaries or a novel pathway. To do this, AKR-2B fibroblasts were treated with a MEK1/2 inhibitor, U0126, 30 minutes prior to the application of TGF-β. Measurement of Erk phosphorylation status showed complete attenuation, indicating that MEK does appear to act upstream of Erk and plays an intregral role in TGF-β mediated Erk activation (Fig. 4D).

Since PAK2 has been previously shown to be specifically activated in fibroblasts and can activate the MAPK pathway (Wilkes *et al.*, 2003), the next step was to determine what role if any, PAK2 has upstream of Erk. We first looked at phosphorylation of c-Raf at Serine 338, a site known to be phosphorylated by PAKs and believed to be critical for c-Raf activation (Diaz *et al.*, 1997;King *et al.*, 1998). In AKR-2B fibroblasts, our results show a time dependant increase in c-Raf

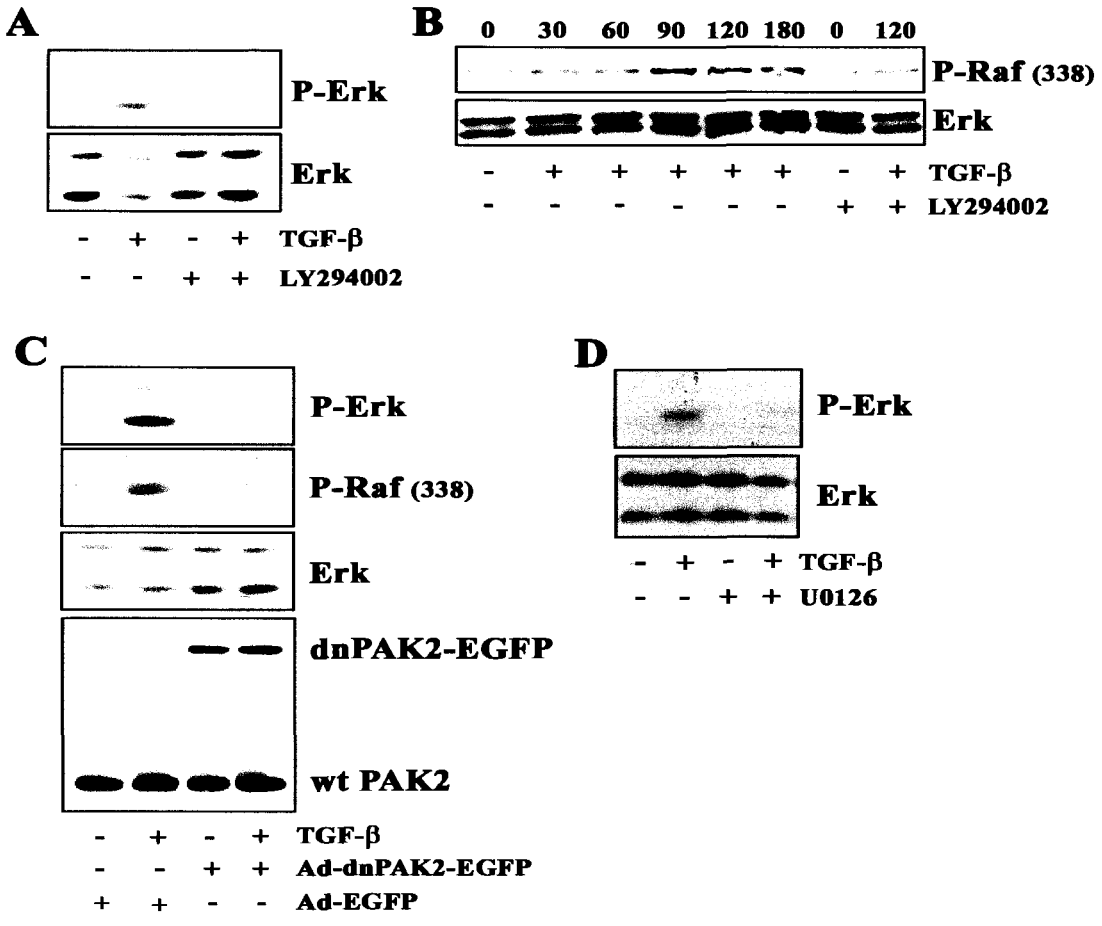

Fig. 4. **Erk is activated in fibroblasts via the PI3K/PAK2/c-Raf/MEK pathway. (A)** AKR-2B fibroblasts were treated with the PI3K inhibitor LY294002 (10μM) 30 minutes prior to addition of TGF-β (2 ng/ml) for 2 hours. Cell lysates were probed with an antibody specific to phospho-Erk, then blots were stripped and reprobed for total Erk as a loading control. **(B)** AKR-2B fibroblasts were treated with TGF-β for the indicated times. Cells were also treated with LY294002 (10μM) 30 minutes before TGF-β (2ng/ml) was added for 2 hours. Cell lysates were probed using an antibody specific to phospho-c-Raf (Ser338), and an antibody to total Erk as a loading control. This loading control was measured using a lower M_r part of the same gel. **(C)** AKR-2B fibroblasts were infected at an MOI of 1:125 with adenovirus containing either dominant-negative PAK2 (Ad-dnPAK2-EGFP) or Ad-EGFP as a negative control. Cells were treated with TGF-β (2ng/ml) for 2 hours prior to lysis. Cell lysates were then probed for phospho-Erk, total Erk, and phospho-c-Raf (Ser338) levels. The loading control was measured using a lower M_r part of the same gel. Lysate was also probed for PAK2 to confirm expression. **(D)** AKR-2B fibroblasts were treated with the MEK1/2 inhibitor U0126 (10μM) 30 minutes prior to addition of TGF-β (2ng/ml) for 2 hours. Cell lysate was probed with an antibody specific to phospho-Erk.

phosphorylation in response to TGF-β. Furthermore, when treated with LY294002, this phosphorylation was negated (Fig. 4B). Additionally, AKR-2B cells were infected with Adenovirus containing enhanced green fluorescent protein (Ad-EGFP) or dominant negative PAK2-EGFP fusion protein (Ad-dnPAK2-EGFP). Phosphorylation levels of c-Raf and Erk were subsequently measured. Expression of EGFP had no effect on the levels of either c-Raf or Erk phosphorylation. Expression of dominant negative PAK2 however, did abrogate both c-Raf and Erk phosphorylation (Fig. 4C). Taken together, these results support the hypothesis that TGF-β induced phosphorylation of Erk is incumbent upon the actions of PI3K and PAK2, through the classic MAPK signaling intermediates.

4.3 Inhibition of Ras propagates Erk activity

The small G-protein Ras has been implicated in the activation of a number of downstream effectors including Erk. In certain cancers, Ras is constitutively active and drives cellular proliferation through the classical MAPK pathway and has been implicated in TGF-β signaling (Leevers and Marshall, 1992;Suzuki *et al.*, 2007). As such, we decided to determine if Ras played a role in TGF-β induced Erk activation. To do this, we treated AKR cells with FPT, a farnesyl transferase inhibitor for 2 hours in the presence or absence of TGF-β. FPT inhibits the ability of Ras to anchor to the membrane, thereby attenuating its function. When Ras activity is inhibited, TGF-β induced Erk phosphorylation was uneffected. In fact, Erk phosphorylation appeared to increase in the

presence of FPT (Fig. 5). Under these conditions, it appears that Ras function negatively regulates Erk activation.

4.4 PI3K/Akt and PI3K/Erk pathways are independent

Akt has been established as a main effector of PI3K signaling. In order to determine the level of interaction between the PI3K/Akt and the newly established PI3K/Erk pathway, AKR cells were treated with LY294002 and U0126 and probed for phospho-AKT. Akt activation was abolished in the presence of the PI3K inhibitor, but no effect was seen in the presence of the MEK inhibitor, indicating that the pathways are independent of one another with respect to direct activation of Erk (Fig 5).

4.5 RNA and protein synthesis are necessary for Erk activation

Since the observed activation of Erk appears around 90 minutes after TGF-β addition, we wanted to examine if there was a required protein that needed to be synthesized first to allow signaling to procede. We therefore treated AKR fibroblasts with Actinomycin D and cyclohexamide, an RNA and protein synthesis inhibitor respectively. When cells were treated with these inhibitors, there was little difference in Erk phosphorylation in the presence or absence of TGF-β, indicating that an essential protein must be produced in order for Erk activation to occur (Fig. 6). Addition of both inhibitors caused an increase in background levels of Erk phosphorylation. This was probably due to an increase in cellular stress upon addition

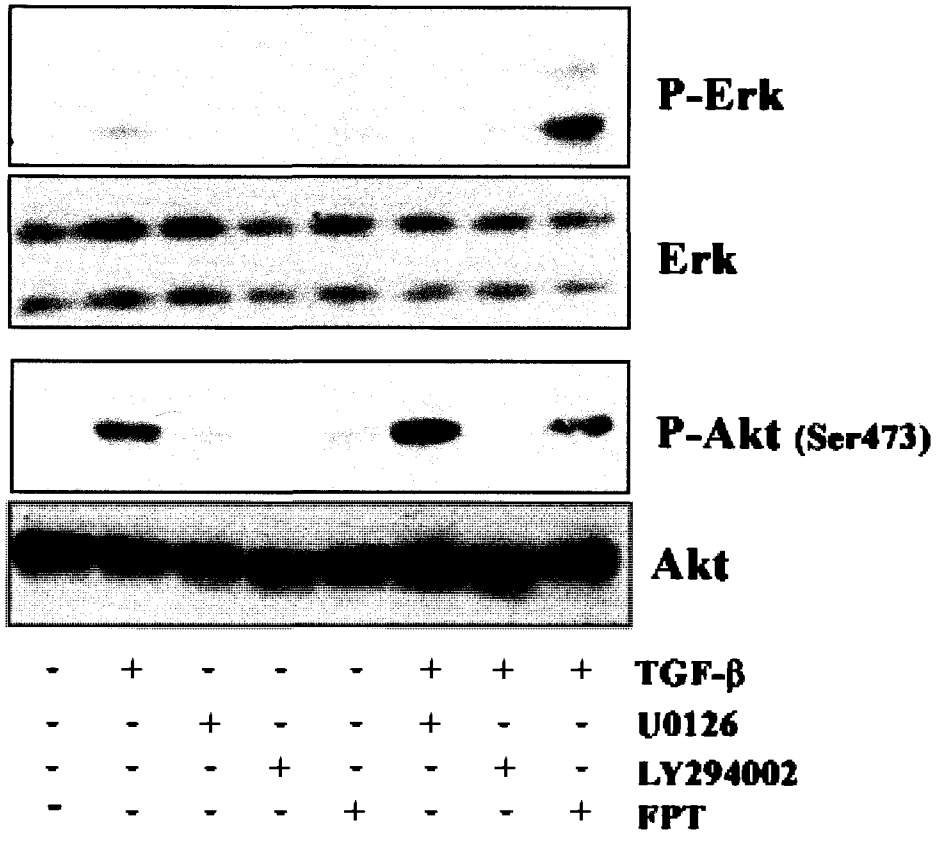

Fig. 5. **Activation of Two Distinct Pathways by TGF-β.** Cell lysates were obtained from AKR-2B fibroblasts treated with TGF-β (2ng/ml) for 2 hours. Separate wells were treated with either U0126 (10μM), LY294002 (10μM), and FPT (10μM) for 30 minutes prior TGF-β treatment. Experiment was performed in triplicate. Cell lysate was probed with antibodies specific to phospho-Erk and phospho-Akt (Ser473). Total Erk and Akt levels are shown as loading controls. Samples treated with or without TGF-β and/or U0126 were also shown in figure 2D

of each inhibitor as Erk as been shown to be activated under conditions of stress (Schliess *et al.*, 1995;Guyton *et al.*, 1996;Pearce *et al.*, 1996).

4.6 Smad2 Linker Region is Phosphorylated by Erk

With the establishment of a direct TGF-β/Erk signaling pathway, our next goal was to determine if an association existed between Erk and the Smad pathway, both under the direct contol of TGF-β. A variety of studies have been undertaken to determine the extent of Erk influence on Smad function. These studies have been unable to clearly define the relationship between Erk and Smad, with Erk phosphorylation of Smad Linker Region sites having been shown to increase Smad activity (Yue *et al.*, 1999) or decrease Smad activity and alter the intracellular trafficking (Kretzschmar *et al.*, 1999). AKR-2B fibroblasts were treated with TGF-β for various times and then probed for Smad2 phosphorylation at the linker region Serine residues 245, 250, and 255. Addition of TGF-β yielded an increase in Smad2 linker region phosphorylation over time. Furthermore, when MEK activity is inhibited prior to TGF-β treatment, a complete abolition of linker region phosphorylation occurs (Fig. 7A). Receptor mediated Smad2 phosphorylation was also measured, showing prominent Serine 465/467 phosphorylation over 3 hours, independent of MEK activity (Fig. 7B). Together this indicates that TGF-β signaling is responsible for phosphorylation at these particular sites within the Smad2 linker region, through activation of Erk.

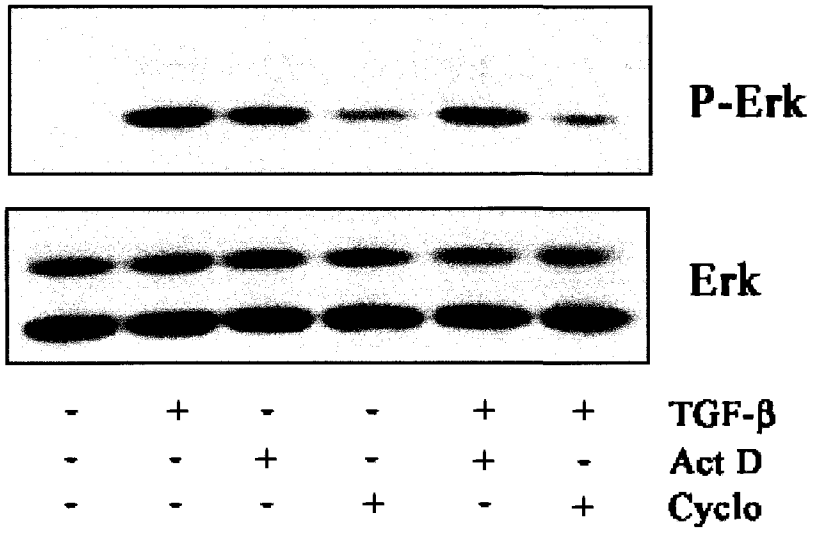

Fig. 6. **RNA/Protein Synthesis is Necessary for Erk Activation.** AKR-2B fibroblasts were treated with TGF-β (2ng/ml) for 2 hours. Actinomycin D (Act D; 2μM) and Cyclohexamide (Cyclo; 10μM) were added 30 minutes prior to TGF-β addition. Cell lysate was probed for phospho-Erk and total Erk as a loading control. Each experiment was performed in triplicate.

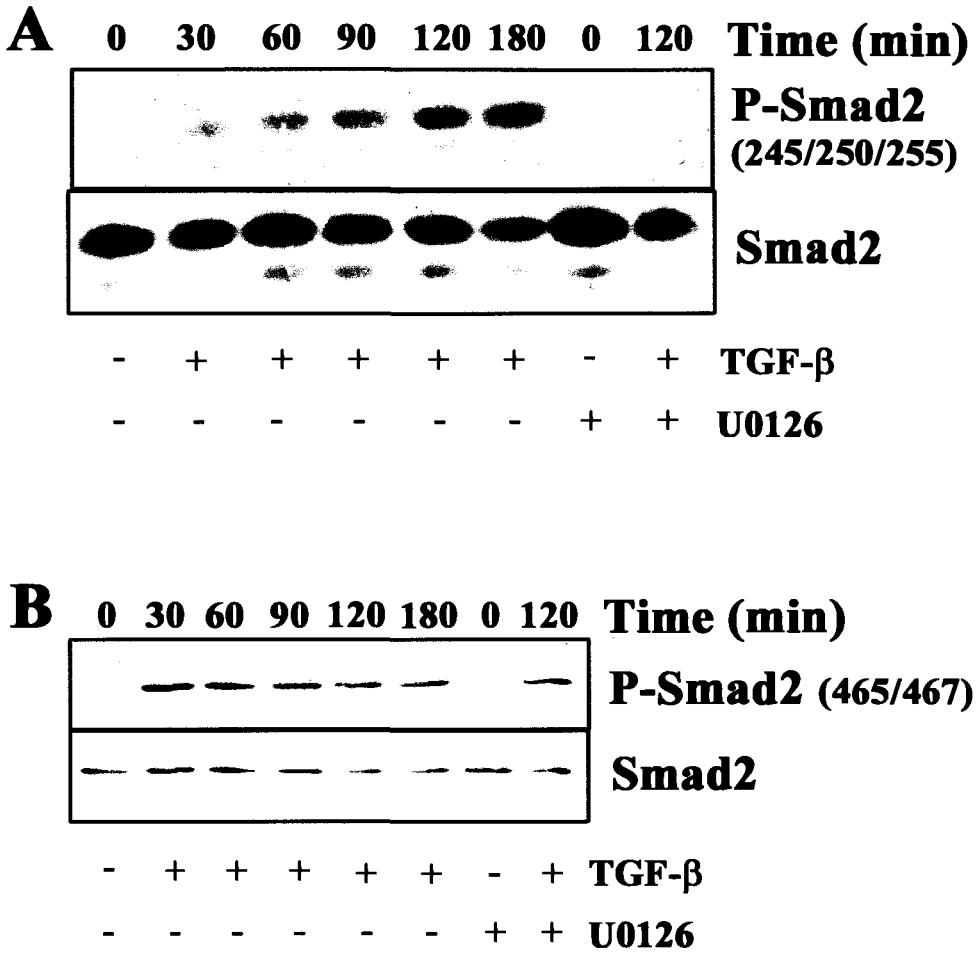

Fig. 7. **TGF-β directs Erk phosphorylation of Smad2 linker region. (A)** AKR-2B fibroblasts were treated with TGF-β (2ng/ml) for the indicated time periods with or without U0126 (10μM) for 30 minutes prior to a 2 hour treatment with TGF-β (2ng/ml). Cell lysates were probed with an antibody specific to Smad2 phosphorylated linker region serine sites 245,250, and 255. **(B)** Receptor mediated phosphorylation of Smad2 was also determined under identical conditions. Cell lysate was probed with antibodies specific to phospho-Smad2 (Ser 465/467). The blots were stripped and reprobed for total Smad2 to demonstrate similar loading of all samples. The experiment were performed in triplicate with consistent results.

These results indicate a direct connection between Smad2 and Erk, but not the functional relationship between the two. Since Smad2 is a transcription factor that translocates to the nucleus following receptor mediated phosphorylation, we fractionated AKR-2B fibroblasts into cytoplasmic and nuclear extracts after addition of TGF-β. We then measured Smad2 linker region phosphorylation. Surprisingly, linker phosphorylation was seen only in the nuclear fraction, not the cytoplasmic fraction (Fig. 8A). Total Smad2 and GAPDH levels were used as controls. As expected, receptor phosphorylated Smad2 was identified predominantly in the nucleus after addition of TGF-β (data not shown). Small amounts were seen in the cytoplasmic fraction, but only in the presence of TGF-β. Total Smad2 was present in both the cytoplasm and the nucleus. In the absence of TGF-β, levels of Smad2 are greater within the cytoplasm. When TGF-β is present, the majority of Smad2 measured was found within the nucleus. Both cytoplasmic and nuclear fractions were probed for GAPDH to show minimal level of cytoplasmic protein contamination of the nuclear fractions (Fig. 8A).

In order to substantiate our findings, immunofluorescent localization of Smad2 linker region phosphorylation was determined. Linker region phosphorylation and localization were similar to the results observed with the cellular fractionations, indicating that Erk phosphorylation of Smad2 linker region occurs in the nucleus of fibroblasts (Fig. 8B). Taken together, these data demonstrate a localization relationship between Erk phosphorylation of the Smad2 linker region which is limited to a specific subcellular location and that this interaction stems from TGF-β induced activation of the PI3K/PAK2/Erk pathway.

4.7 Nuclear Smad2 is Controlled by the 26S Proteasome in fibroblasts

26S proteasomal activity is an integral part to many signaling systems (Ciechanover, 1998). It provides a mechanism by which signaling pathways can be controlled. When AKR fibroblasts were treated with the proteasome inhibitor MG132, in addition to TGF-β, we found that nuclear levels of Smad2 increase, indicating that the proteasome is involved in the downregulation of Smad2 levels in mammalian fibroblasts (Fig. 8A). This finding was further verified using NIH/3T3 fibroblasts stained for linker region Smad2 phosphorylation (Fig. 8B). When proteasome activity was inhibited prior to TGF-β stimulation, there is a large increase in Smad2 linker region phosphorylation, suggesting that the proteasome has a role in the control of TGF-β signaling through degradation of Smad2. This data is similar with previous work showing Smad2 to be under the control of the 26S proteasome (Lo and Massague, 1999).

4.8 Erk Function is Critical for Proliferation in Fibroblasts

Erk functions to phosphorylate a variety of cytoplasmic and nuclear targets, many of which are critical in cell cycle progression (Gille *et al.*, 1995;Weber *et al.*, 1997;Sears *et al.*, 1999;Sears *et al.*, 2000). Having established a connection between Erk and the Smad signaling pathway, we sought to determine if Erk also plays a role in the proliferative effects of TGF-β in fibroblasts. As such, we focused on the proto-oncogene c-*myc*, known to be mutated in a variety of cancers and an important promoter of cell growth. Figure 9A shows that when AKR-2B fibroblasts were treated with TGF-β, c-*myc* phosphorylation increases over a similar time course to that seen with Erk activation (Fig.

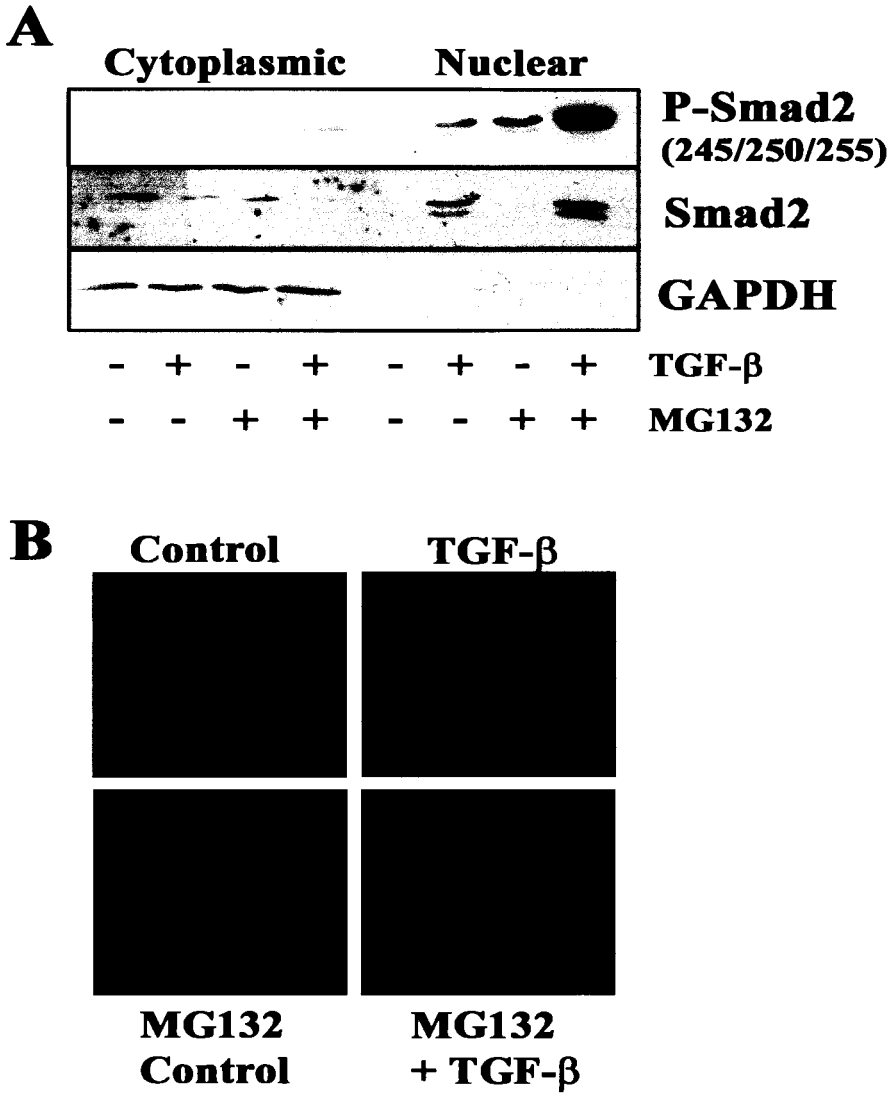

Fig. 8. **Nuclear Smad2 Levels are Controlled by the Proteasome. (A)**AKR-2B fibroblasts were treated for 3 hours with TGF-β (2ng/ml). MG132 (10 μM) was added 30 minutes prior to TGF-β addition. Nuclear and cytoplasmic fractions were probed for linker region phosphorylation (phospho-Smad2 (Ser245/250/255)), total Smad2 as a loading control, and GAPDH to monitor the presence of cytoplasmic protein in the nuclear fraction. **(B)** Photomicrographs of NIH 3T3 fibroblasts treated with TGF-β (2ng/ml) for 3 hours. MG132 (10μM) was added 30 minutes prior to TGF-β treatment. Cells were incubated with phospho-Smad2 (Ser245/250/255) antibody and specific immune complexes detected using Rhodamine X conjugated secondary antibody. Experiments provided consistent results and were performed in triplicate.

3A). When TGF-β induced Erk activition is inhibited, c-*myc* phosphorylation is abolished.

In addition to c-myc activation, Erk has been shown to coordinate activation of several proteins to stimulate cell replication. The biological consequences of TGF-β induced Erk activation were addressed using a Thymidine Incorporation assay (Fig. 9B). Treatment with TGF-β yielded a 6 fold increase in DNA synthesis as compared to untreated NIH3T3 fibroblasts. When cells were treated with U0126 to inhibit Erk activation prior to TGF-β addition, growth was attenuated 6 fold when compared to cells treated with TGF-β alone. This is significant in that the abolishment of Erk activation with U0126 completely diminished TGF-β induced growth proliferation in the NIH 3T3 fibroblast cell line. Consistent with our previous data showing PAK2's role in Erk activation, the presence of dnPAK2 dramatically decreased TGF-β stimulation to only 2 fold above control levels (Fig. 9B). Thus, the ability of TGF-β to induce growth in fibroblasts appears to depend on the function of Erk and PAK2.

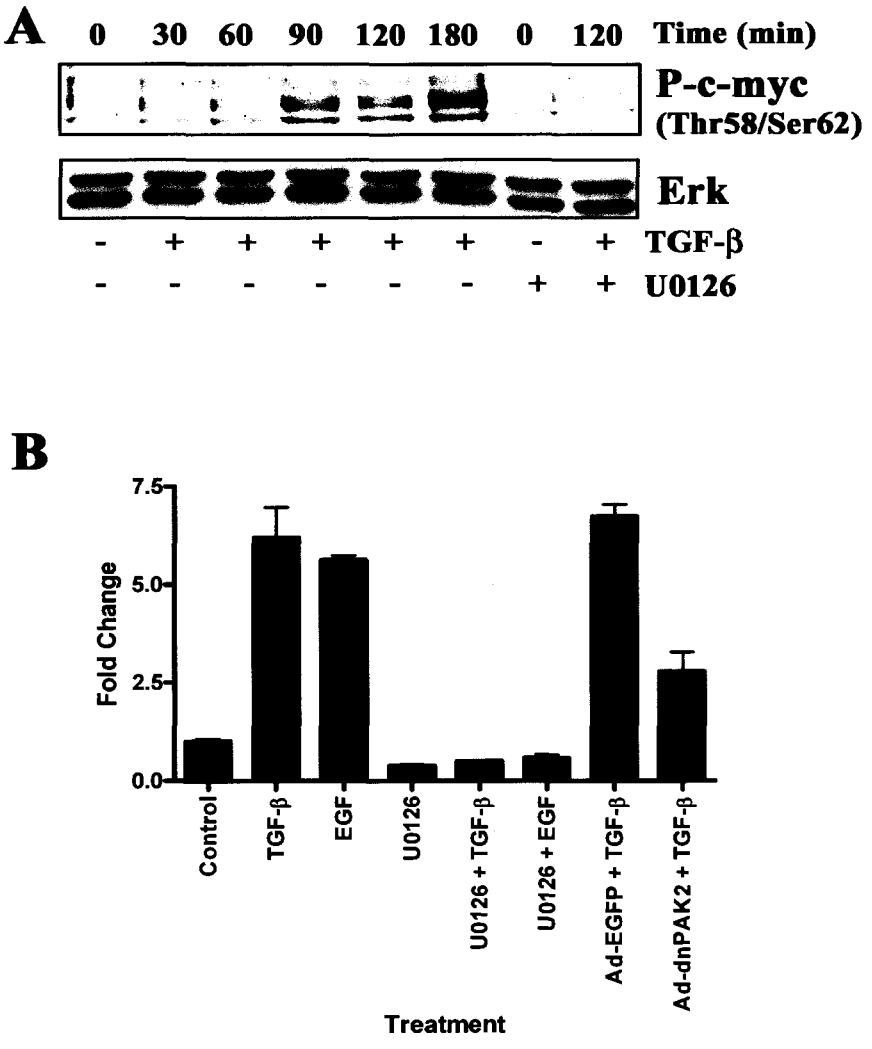

Fig. 9. **Erk Activity is Integral for TGF-β Induced Growth in Fibroblasts. (A)** Cellular lysates of AKR-2B fibroblasts were obtained from cells treated with TGF-β (2ng/ml) for the indicated times, with or withoutU0126 (10μM) for 30 minutes prior to treatment of TGF-β for 2 hours. Cell lysates were probed for phospho-c-Myc (Thr58/Ser62) and total Erk as a loading control. **(B)** To test the effects on cell growth, a thymidine incorporation assay was performed. Serum deprived NIH 3T3 cells were treated with TGF-β (5ng/ml), EGF (50ng/ml) or infected with Ad-dnPAK2 or Ad-EGFP (MOI = 125:1) prior to treatment with or without TGF-β. The effect of treatment is expressed as a fold change relative to untreated cells (control=1). Each sample was performed in triplicate. The values represent the average of all replicates for each sample.

CHAPTER 5 – Discussion

5.1 Cell-type specific Erk activation

TGF-β has been shown to mediate the activation of a number of downstream targets including Akt, ROCK, and MAPKs, such as Jnk and Erk (Mucsi *et al.*, 1996;Atfi *et al.*, 1997;Bakin *et al.*, 2000;Bhowmick *et al.*, 2001). However, a major limitation in our understanding of TGF-β biology is the lack of knowledge regarding a direct link between TGF-β signaling and the mechanisms and actions of Smad-independent pathways. Considering the functional diversity TGF-β displays, the non-smad pathways of TGF-β signaling have the potential to play a major role either as direct alternative signaling pathways or in crosstalk with smads, to generate the multitude of observed TGF-β effects. It is for these reasons we chose to address the mechanisms involved in direct TGF-β activation of Erk, as well as its functional role in TGF-β signaling in non cancerous cells.

Four phenotypically normal cell lines (2 fibroblast and 2 epithelial) were treated with TGF-β over a period of time. TGF-β was only able to induce Erk phosphorylation in fibroblasts, not epithelial cells (Fig.3). It is important to note that this activation occurs with endogenous levels of all members of the signal transduction pathway demonstrating this is a normal response and not related to over-expression artifacts. This data is consistent with an earlier study indicating that TGF-β induced activation of this non-smad pathway does not take place in epithelial cells and appears to be a cell-type specific phenomenon (Hayashida *et al.*, 2003). As a known effector of cell replication, it is

interesting to note that Erk is activated in a cell type known to proliferate in response to TGF-β, but not in a cell type growth inhibited by TGF-β.

5.2 Erk phosphorylation occurs via PI3K/PAK2/Raf-1 but not Ras

PI3K has been implicated in TGF-β signaling as an activator of PAK2 signaling (Wilkes *et al.*, 2005). Here we show that PI3K also acts downstream of the TGF-β receptor complex to induce the activation of Erk. Abrogation of PI3K function using specific chemical inhibitors greatly reduces Erk phosphorylation (Fig. 4A). Additionally, loss of PI3K function also led to an abolishment of c-Raf phosphorylation at Serine 338, a known site of PAK activation (Diaz *et al.*, 1997). Consistent with the description of the pathway is that dnPAK2 is able to block both c-Raf and Erk phosphorylation induced by TGF-β (Fig. 4B-D). These findings are consistent with previous reports showing other group A PAKs interacting with and phosphorylating c-Raf at Serine 338 (King *et al.*, 1998;Chaudhary *et al.*, 2000). Furthermore, this pathway follows the same trend as the classic MAPK signaling pathway. However, instead of Ras as the primary activator, c-Raf activation appears to occur through PI3K/PAK2, similar to that described for PDGF (Beeser *et al.*, 2005). It appears as though these results follow a similar trend to that found in a previous study whereby PAK2 mimics its budding yeast homologue Ste20 by acting as a MAP4K in a mammalian system (Dan *et al.*, 2001).

The interplay between Ras and the various forms of Raf in forming the initial steps of the MAPK signaling pathways, as well as the interactions between the various Raf isoforms themselves is complex and not completely understood. Ras has been shown

to activate both b-Raf and c-Raf (Troppmair *et al.*, 1992). Oncogenic Ras has been shown to strongly regulate b-Raf, whereas Raf-1 (c-Raf) requires the actions of Ras, in addition to other signals, to activate its tyrosine kinase (Marais *et al.*, 1997). As has been previously indicated for PDGF signaling (Beeser *et al.*, 2005), it is possible that within mammalian fibroblasts, TGF-β induces Erk activation through PI3K separately from Ras. Furthermore, B-Raf appears to contribute to the majority of MEK activation, via Ras (Jaiswal *et al.*, 1994). It is possible that by inhibiting the Ras pathway, the ability of the PI3K/PAK2/c-Raf pathway to initiate Erk phosphorylation is increased.

The role that Ras plays in Erk activation through TGF-β signaling is unclear. Here we show inhibiting Ras appears to increase Erk activation while previous studies have suggested a positive role for Ras in TGF-β/MAPK signaling (Suzuki *et al.*, 2007). One possible explanation may be RLP (Ras-like-protein), a Smad3-dependent immediate-early TGF-β target gene. It shares 30% sequence homology with members of the Ras superfamily and has been shown to interact with type I and type II receptors (Piek *et al.*, 2004). In addition to its similarity with Ras family members, another interesting finding was RLP activation time. Expression was induced within 45 minutes of TGF-β introduction, similar to the observed activation time of Erk, occurring between 60-90 minutes after TGF-β addition. If RLP imitates Ras in terms of acting as a docking site for MAP4Ks and allows for their activation, then RLP may act upstream of Erk and play a role in its activation. The data presented in this report is based on chemical inhibition of Ras. It would be interesting to see if introducing a dominant negative form of Ras would have the same effect. Such an experiment would rule out any secondary effects from

chemical inhibition which may contribute to this phenotype. This approach would also allow us to define in more detail, the mechanisms involved in Erk activation. By mutating the domain which interacts with Raf-1, we could determine if direct Ras-Raf-1 interaction is necessary for Erk activation or if Ras predominantly works through another intermediate such as PI3K.

Furthermore, figure 6 showed that RNA and protein synthesis were necessary to induce Erk activation in the presence of TGF-β. RLP, and possibly other immediate-early genes upregulated by TGF-β, may play a role in Erk activation. This however, is not direct evidence that an unknown protein is involved upstream of Erk activation. It is possible that this a secondary effect whereby Erk is stimulated after activation of other factors which then propagate a signal for Erk activation. This would provide a possible explanation for the time lag seen in Erk phosphorylation.

5.3 Smad2 is phosphorylated by Erk in the nucleus

The treatment of fibroblasts with TGF-β induced phosphorylation of the Smad2 linker region, a site believed to be important in regulating Smad function (Yue *et al.*, 1999;Kretzschmar *et al.*, 1999). In addition, inhibition of Erk activity subsequently abolished this phosphorylation. Previous studies have addressed the potential link between MAPKs and Smad signaling, however an unambiguous definition of the role it plays still remains elusive. Kretzschmar et. al, (1999) showed the ability of oncogenic Ras and EGF to stimulate Erk/Smad interaction resulting in nuclear exclusion of Smad2/3. Our results appear to be contradictory to this report. With both cellular

fractionation and immunocytochemistry displaying linker phosphorylation occuring primarily within the nucleus. The incongruity between our data and previous reports could lie with the fact that previous studies looked in epithelial cells and the interactions of other growth factors with TGF-β, while our study defined fibroblast signaling with TGF-β alone. It is possible that differential affects are due in part to mechanistic differences between cell types, or it may simply be an issue of timing and influence of EGF on smads. Kretzschmar et. al, (1999) observed nuclear exclusion in a system when EGF and Ras activate greater than normal levels of Erk before Smad activation. With a constitutively active Ras, active Erk is present before and after TGF-β activation of Smads. Our study is focused on cells functioning in a context of TGF-β alone whereby Smads are activated immediately and begin to translocate into the nucleus. Once activated approximately 60-90 minutes later, Erk has to move into the nucleus, where most of the Smad2 is already, to phosphorylate and affect its function.

The apparent localization of linker region phosphorylation within the nucleus raises a number of interesting questions regarding the role of Erk/Smad signaling kinetics under TGF-β signaling. Phosphorylation of Smads by TGFβR-I occurs approximately 5-15 minutes after TGF-β introduction (data not shown). Interestingly, Erk phosphorylation was not seen until approximately 60-90 min. after TGF-β induction. This would mean that Smads would be functioning for approximately 45-60 min. before any substantial activation of Erk is seen. Taking this into account, it is possible that a function of Erk may be as an inhibitor of Smad function. The time difference before Erk activation would allow for Smads to function uninhibited before being constrained by direct or indirect Erk

interactions. A recent report introduced the idea that complexed Smad2/4 molecules are retained in the nucleus and uncomplexed smads are shuttled back into the cytoplasm to be phosphorylated by TGFβRI, oligomerize, then shuttle back into the nucleus to be retained (Nicolas *et al.*, 2004;Schmierer and Hill, 2005). It is possible that Erk may only phosphorylate the linker region of Smad2 and affect its function when it is complexed and active within the nucleus. Lo et al. (2002) showed that Smad2 signaling appeared to be controlled by ubiquitin dependent degradation via the proteasome within the nucleus in a number of different cell types. We corroborated these findings by showing that the proteasome does regulate nuclear Smad2 levels in both AKR-2B and NIH/3T3 fibroblasts. It would be interesting to determine if there is a correlation between Smad2 linker region phosphorylation by Erk and Smad2 degradation. Smad2 signaling may be attenuated upon Erk activation. The down regulation of the Smad pathway coupled with the increase of proliferative signals such as c-Myc, could explain the dependence of Erk activity for proliferation in fibroblasts as demonstrated in figure 9.

5.4 Erk controls fibroblast proliferation

In addition to its interaction with the Smad signaling pathway, Erk also acts to directly affect growth in fibroblasts. Figure 5B illustrates the importance of Erk in the TGF-β growth proliferative effects seen in fibroblasts as U0126 clearly inhibits TGF-β cell growth. This data is in line with previous reports illustrating the ability of Erk to upregulate cyclin D1 transcription and downregulate Cdk inhibitor $p27^{KIP1}$ expression, leading to cell cycle progression (Weber *et al.*, 1997). In addition to its effects on cyclin

D1 and $p27^{KIP1}$, Erk also activates a number of transcription factors, including the proto-oncogene c-myc (Davis, 1995) and increases its stability via phosphorylation (Sears *et al.*, 2000). Overexpression of the Myc protein has been cited in many different types of cancer, including breast and ovarian cancers (van Dam *et al.*, 1994). As expected, our results indicate that Erk activity induces c-Myc phosphorylation at Ser58/Thr62(Fig 9A). This is significant in that we are able to show a direct link between the activated TGFβR complex and a non-smad transcription factor and proto-oncogene involved in the cell proliferation process. Furthermore, Erk may also indirectly effect c-myc activity through the control of Smad signaling. An earlier study reported a link between the Smad pathway and the c-myc promoter (Yagi *et al.*, 2002). Conversely, TGF-β has been implicated in the down-regulation of c-myc in epithelial cell types (Pietenpol *et al.*, 1990). Taking this data into consideration, it is possible that in addition to phosphorylating c-myc, Erk inhibition of Smad signaling may help to increase c-myc expression. As such, the data presented may offer a possible explanation for the differential effects TGF-β displays between cell types.

Although Smad-dependant signaling has been well defined, Smad-independent signaling is not well understood. The purpose of this study was to define the mechanisms involved in the TGF-β induced activation of Erk. Our results indicate that with TGF-β stimulation the MAPK, Erk, is activated in a cell type specific manner. TGF-β induces the phosphorylation of Erk through the PI3K/PAK2 dependant pathway. Abolishment of PAK2 activity inhibits c-Raf and Erk phosphorylation under TGF-β stimulation, indicating that TGF-β functions through PAK2 to activate c-Raf and subsequently Erk.

Furthermore, the MAPK phosphorylation sites found within the linker region of Smad2 are phosphorylated by Erk and this phosphorylation appears to only occur within the nucleus. The action of Erk is integral to the growth effects of TGF-β in fibroblasts. Abrogation of Erk activity leads to a blockade of TGF-β induced stimulation of growth (Fig. 9).

The data presented here, in addition to published reports, allows us to hypothesize a possible mechanism of action for TGF-β in fibroblasts. First, Smads are immediately activated upon TGF-β induction and proceed to regulate target gene transcription until Erk is activated approximately 60 min later. Erk then abolishes the overall negative effects on growth conferred by Smads either through direct or indirect regulation of Smad signaling and by upregulating transcription factors such as c-myc to induce cell cycle progression. Taken together, these results indicate an important and multi-faceted role for Erk in TGF-β signaling. Elucidation of this pathway has shed light on key questions pertaining to TGF-β pathway interactions, as well as mechanistic differences between cell types. By doing so, we are able to gain insight into the role it plays in cancer and other diseases.

5.5 Future Directions

The data presented here provide a basis for study of the interplay between various facets of TGF-β signaling. A major hurdle in understanding TGF-β and its role in cancer is first understanding how it functions in a normal environment. Delineating the

mechanisms involved in Erk activation through TGF-β contributes to our overall understanding of the system, but there are still many questions to be answered.

The role of Ras in the process of Erk activation remains elusive. It appears as though Ras has a negative effect on the degree of Erk activation, but this has only been studied briefly using chemical inhibitors during this study. Recent published data has suggested a positive role for Ras in the activation of downstream effectors of TGF-β (Suzuki *et al.*, 2007). It would be interesting to use dominant negative or constitutively active forms of Ras and measure the effect has on downstream signaling after TGF-β stimulation. Moreover, the mutation of certain sites within Ras or its effectors such as Raf-1 or PI3K that effect interaction would be interesting to see what role each played in Ras signaling. This would provide a clue as to the mechanism at play further upstream of Erk.

We have shown a direct link between Erk and Smad2. Erk phosphorylates the linker region of Smad2, but the functional purpose of this phosphorylation has yet to be determined. Previous studies have shown either a negative effect on smad signaling through inhibition of smad nuclear translocation or a positive effect through gene regulation. This incongruency between studies may be cell-type specific or the phosphorylation may be multifunctional. Our data does not specifically disagree with the theory of smad nuclear inhibition. We show that Erk activation by TGF-β does not block nuclear export. Other studies have shown what other growth factors, such as EGF, which activate Erk, do to R-smad translocation. We have however not studied the difference in gene regulation between smads phosphorylated or not phosphorylated at the linker

region. It would be interesting to study a subset of genes and their activity when Erk is active or inactive to determine if there is a difference. Furthermore, the role of the proteasome has yet to be fully defined in smad signaling. Smad levels have been shown to be regulated by the proteasome. However, the signals which target smad for degradation are not known. When the proteasome is inhibited via MG132, the levels of Smad2 are greatly increased in the nucleus. We also showed that linker region phosphorylation seems to only occur within the nucleus. We hypothesize that Erk phosphorylation of the Smad2 linker region could be the signal which directs Smad2 for degradation by the proteasome. With Smad signaling having an overall negative effect on cell proliferation, it seems plausible that Erk, a major effector of proliferation, could dampen the negative effects of smads while stimulating factors involved in pushing the cell through the cell cycle such as c-myc. Mutating serines 245, 250 and 255 and measuring Smad2 levels in the nucleus after Erk activation would allow us to see if phosphorylation of these sites by Erk was necessary for degradation. Furthermore, measuring the amount of ubiquitination of Smad2 when Erk is active and when it is inhibited would also give a good indication of the role of Erk in Smad2 regulation.

The data we present in this study provides insight into TGF-β signaling in fibroblasts. Our study helps to explain why there is a cell-type specific difference in TGF-β signaling as well as explaining the role smad-independant signaling plays. However, our knowledge in this field is still very limited. The results generated here will help to explain various facets of TGF-β signaling and will allow further progression in the study of its role in cancer.

5.6 Summary

The main purpose of this study was to assess the factors involved in smad-independent signaling under TGF-β control within phenotypically normal cells. Here we show a cell-type specific TGF-β controlled activation of Erk. Cells of mesenchymal origin appear to initiate this activation through the PI3K/PAK2/c-Raf/MEK pathway. There appears to be a temporal delay in Erk phosphorylation that occurs approximately 60-90 minutes after TGF-β addition. Furthermore, Erk activation appears to affect cell proliferation, potentially through the control of transcription factors such as c-myc, as well as through cessation of the Smad signaling pathway.

The MAPK, Erk, has been shown to potentiate cellular growth and proliferation by activating or deactivating a number of substrates (Davis, 1995;Weber *et al.*, 1997). As such, Erk activation in fibroblasts and their ability to proliferate in the presence of TGF-β appear to coincide. When Erk activation was inhibited growth appeared to be attenuated as measured by thymidine incorporation. Furthermore, we showed a direct interaction between Erk activation and linker region phosphorylation of Smad2 in the nucleus. The exact purpose of this interaction has not been fully elucidated. However, Smads do appear to have an overall negative effect on growth. It would be reasonable to assume that in addition to stimulating proliferation through activation of various effectors, Erk may also be promoting proliferation by controlling the anti-proliferative signals of Smads.

These potential roles for Erk would help explain the effects of TGF-β seen in various cancers and other disease states. In normal epithelial cells, long term (days)

treatment with TGF-β induces a change in phenotype more characteristic of mesenchymal cells. This is known as epithelial-mesenchymal transition (EMT) (Miettinen *et al.*, 1994). This transition includes the ability of epithelial cells to proliferate under TGF-β control. It is possible that the subsequent ability to proliferate may stem from the activation of Erk. In order for cells to proliferate in the presence of TGF-β, the actions of Smads must be abrogated. Our results have shown a direct interaction between Erk and Smads. It is possible that in some way, Erk does directly control Smad activity. Furthermore, the activation of PI3K and its subsequent activation of both Erk and Akt drive cellular proliferation. The data presented here could help explain how cells make the transition from growth inhibition to proliferation in the presence of TGF-β.

The pathways initiated by TGF-β independent of the Smad pathway, are poorly understood. There is little definitive data for these Smad-independent pathways and how they contribute to TGF-β phenotypes in varying cell types. With the multitude of effects TGF-β portrays, it would be surprising to learn that all stem from Smad signaling. As such, it is important to characterize the mechanisms and function of these Smad-independent pathways. By defining these pathways and learning how they function in a normal system, we are better able to understand what may be going on under aberrant conditions.

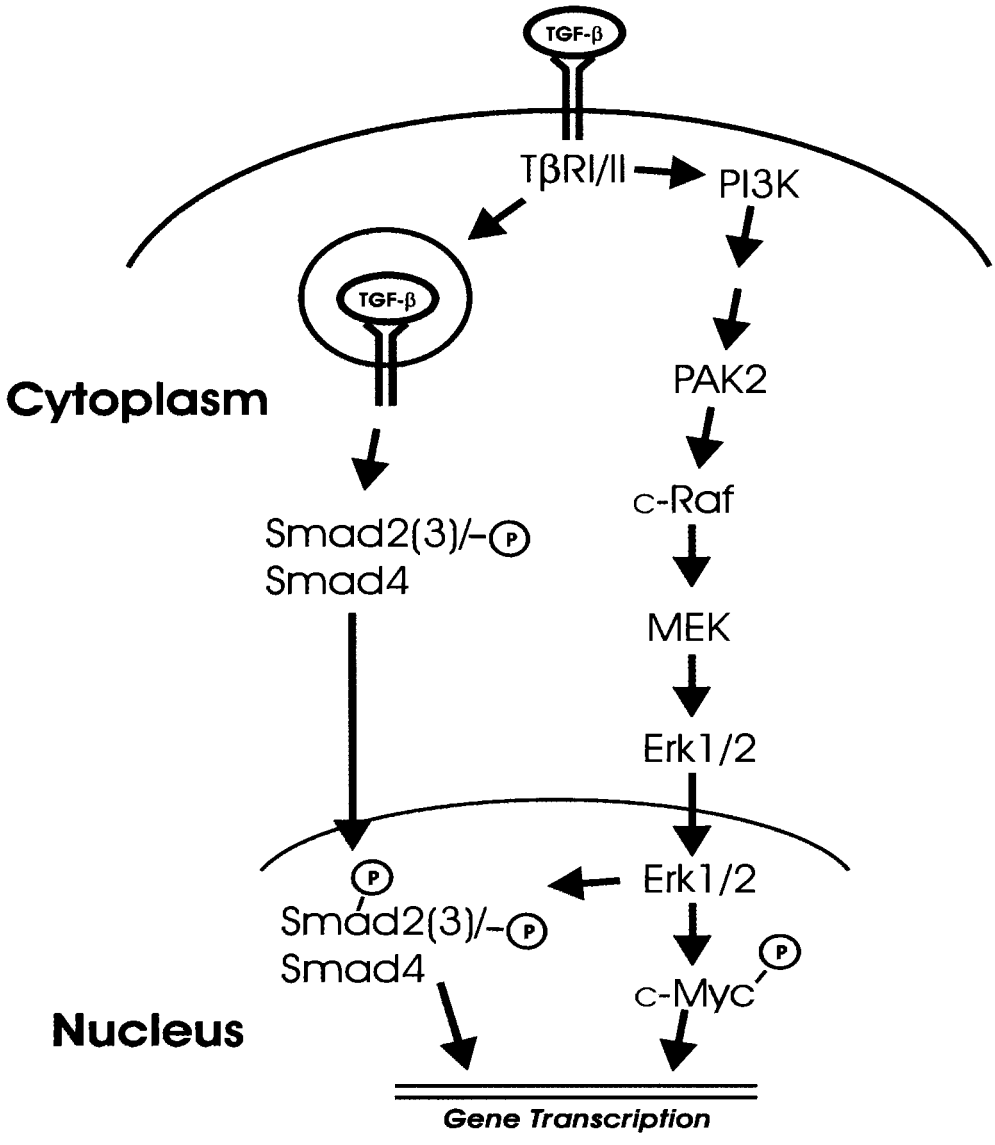

Fig. 10. **Mechanisms of TGF-β Induced Erk Activation.** A schematic diagram depicting a proposed mechanism by which TGF-β causes Erk phosphorylation through the PI3K→PAK2→c-Raf→MEK pathway. Erk shows potential regulation of Smad2 signaling by phosphorylating its linker region within the nucleus and may initiate potent growth stimulatory signals via transcription factors such as c-Myc. Arrows do not necessarily depict a direct link between each protein.

CHAPTER 6 - References

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