TRANSFORMING GROWTH FACTOR BETA REGULATES CELL CYCLE AND GROWTH VIA AKT

DANIELLE V. KIELLEY

Transforming Growth Factor Beta Regulates Cell Cycle and Growth via Akt

Danielle V. Kielley

Faculty of Medicine
Memorial University of Newfoundland

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Abstract

Research Question: Transforming growth factor beta (TGF-β) causes growth stimulation and transformation in fibroblasts, but growth inhibition/apoptosis in other cell types. Previously, TGF-β has been shown to activate the Smad signalling cascade in all cell types. Alternative signalling pathways have been described in response to TGF-β. To explain how TGF-β controls growth, we investigated a downstream target of phosphatidylinositol 3-kinase (PI3K) called Akt. Akt inactivates glycogen synthase kinase 3 beta (GSK-3β) and FOXO. We propose that PI3K signalling is partially responsible for the different phenotypic effects of TGF-β in mesenchymal and epithelial cells.

Methods: Western blotting was used to describe temporal changes (0-3 hours) in PDK-1, Akt, GSK-3β and Cyclin D1 phosphorylation/protein levels with TGF-β2 stimulation of normal fibroblast and epithelial cell lines. Additionally, we blocked the effects of TGF-β on Akt/GSK-3β using PI3K/Akt specific inhibitors and TGF-β receptor kinase dead cell lines. Using the UAS/GAL4 system of ectoptic gene expression in *Drosophila melanogaster*, we overexpressed the activated Baboon receptor (Activin/TGF-β type I homologue) in an eye-specific manner, using early and late developmental drivers. We also coexpressed GSK-3β, $PI3K^{DN}$ and Akt constructs with activated Baboon. Results were documented using Scanning Electron Microscopy (SEM).

Results: TGF-β stimulation increased PDK-1, Akt and GSK-3β phosphorylation in fibroblasts over time. Conversely, TGF-β stimulation decreased PDK-1, Akt and GSK-3β phosphorylation over time in epithelial cells. The GSK-3β substrate, Cyclin D1, exhibited

decreasing phosphorylation in fibroblast cells, and decreased protein levels of Cyclin D1 in epithelial cells. In fibroblasts, TGF-β increased Cyclin D1 complex formation with CDK-4 and nuclear localization. Conversely, TGF-β decreased complex formation in epithelial cells. TGF-β stimulated Cyclin D1/CDK complex formation was blocked by a PI3K inhibitor. Morphological transformation by TGF-beta was attenuated by an Akt inhibitor in fibroblasts. In Drosophila studies, overexpression of constitutively active Baboon in the eye caused overgrowth and patterning defects that was rescued by coexpressing either a $PI3K^{DN}$ mutation, mutant dFOXO or mutant GSK-3β, while overexpression of wild-type Akt or PI3K had a synergistic effect.

Summary: These results, in both cell culture and Drosophila, point to a novel mechanism of TGF-β signaling through PI3K/Akt. In total, these results define a novel pathway for TGF-β regulation of cell cycle through PI3K/Akt in normal cells. This signalling pathway also differentiates epithelial and fibroblast cell responses to TGF-β stimulation. In conjunction with Smads, this pathway could account for the cell type specific growth responses to TGF-β. Potential alterations of this pathway may be a mechanism by which some cancers elude the normal growth regulatory system imposed by TGF-β.

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List of Abbreviations

Dakt	= Drosophila Akt
Bad	= Bcl-2 pro-apoptotic family member
BMP	= Bone Morphogenetic Protein
chico	= Gene encoding a insulin receptor substrate (Drosophila)
cdc24	= Guanine-nucleotide dissociation stimulator
CDK	= Cyclin Dependent Kinases
Daf	= Dauer Defective
dFOXO	= Drosophila Forkhead transcription factor, sub-family "O"
DILPs	= Drosophila insulin-like peptides
DInr	= Drosophila Insulin and IGF receptor
DMEM	= Dulbecco's modified eagle serum
dpp	= decapentaglegic
DPTEN	= Drosophila Phosophatase and Tensin Homologue on chromosome ten
EGF	= Epidermal growth factor
EMT	= Epithelial to mesenchymal transition
ERK	= Extracellular regulated kinase
FBS	= Fetal Bovine Serum
FOXO	= Forkhead transcription factor, Forkhead box subfamily "O"
GADD45	= Growth arrest and DNA-damage inducible
GAL4	= Yeast transcription enhancer protein
GDF	= Growth and differnation factors
GDNF	= Glial cell-line derived neurotrophic factor

GMR = Glass multiple reporter (eye development)

GS Domain = Cytoplasmic jutxamembrane region containing a serine-glycine repeat regions

GSK-3α = Glycogen Synthase Kinase Alpha

GSK-3β = Glycogen Synthase Kinase Beta

GMR = Glass multiple reporter (eye development)

IGF = Insulin-like growth factor

ILK = Intergrin Linked Kinase

IKK =NFκB-kinase α

IRS1-4 = insulin receptor substrate 1-4

JNK = Jun N-terminal kinase

LIMK1 = LIM kinase1

MAPK = Mitogen activated protein kinase

Mv1Lu = mink lung epithelum

NBCS = Normal Bovine Calf Serum

NMuMG = mammary gland epithelium

NGF = nerve growth factor

NRK-49 = Normal Rat Kidney Cells

PAK-2 = p21 Activated Kinase

PBS = Phosphate Buffer Saline

PDGF = platelet derived growth factor

PDK-1 = Phosphoinositide-dependent kinase-1

PH = Plectsrin homology domain

PI3-K = phosphatidylinositol 3-kinase

PtdIns = phosphatidylinositol

PtdIns(4)P = phosphatidylinositol (4) P

PtdIns(4,5)P_2 = PtdIns(4,5)P_2

PtdIns(3)P = phosphatidylinositol 3-phosphate

PtdIns(3,4)P_2 = phosphatidylinositol (3,4) bisphosphate

PtdIns(3,4,5)P_3= phosphatidylinositol (3,4,5) triphosphate

p85 = regulatory subunit of PI3K

p110 = catalytic subunit of PI3K

Rac1 = Rho family, small GTP binding protein

RAS = GTP binding protein

RhoA = Ras homolog gene family, member A

RTKs = receptor tyrosine kinase receptors

SEM = Scanning Electron Microscopy

Ser = Serine

SGK = Serum glucocortiod kinase

SH2 = Src homology 2 domain

SARA = Smad anchor for receptor activation

SMURF = Smad ubiqutination regluatory factor

TGF-β = Transforming growth factor beta

Thr = threonine

UAS = Upstream activation sequence

w^{1118} = $White^{1118}$ (Control flies)

WNT = wingless-type MMTV integration site family

Chapter 1: Introduction

Chapter 1.1: Introduction to Cellular Growth

The Transforming Growth Factor Beta (TGF-β) superfamily of peptides is comprised of key regulators of growth in both developing and differentiated cells (Massague *et al.*, 2000). However, the mechanisms by which this family of cytokines control cellular growth are not clear, causing growth inhibition in some cell types such as epithelial and immune, growth stimulation in others, including fibroblasts and a cytostatic signal to neurons and other cell types (Massague, 2000). There are two primary mechanisms of cellular growth: 1) increases in cell size and 2) increases in the rate of cell division. Cellular growth is influenced by extracellular signals that influence identity, pattern, structure and final size of the organ in development. Growth in development is controlled by the Wingless/Wnt and TGF-β [(Bone Morphogenetic Protein (BMP)/Decapentaplegic (Dpp)] pathways (Johnston and Gallant, 2002). Collectively, these pathways result in the modulation of various genes and their respective proteins to control metabolic functions that regulate the rate of growth and the differentiation of a given cell to ultimately determine the type and size of an organ during development.

In this project, the prototypic member of the TGF-β superfamily, TGF-β and its role in cellular growth signalling pathways was investigated. The best characterized signal transduction pathway of TGF-β action is the Smad pathway (Shi and Massague, 2003). All categories of cells appear to activate identical receptors and the same downstream intracellular pathway (Smads), in response to a ligand despite TGF-β exerting different effects on the same cell types, at different times in development. There

are several 'alternative' pathways such as the Ras/MAPK, phosphatidylinositol 3-kinase (PI3K)/p21 activated kinase 2 (PAK 2) and the PI3K/Akt pathways, have been described (Mulder and Morris, 1992;Mulder, 2000;Shin *et al.*, 2001;Yu *et al.*, 2002;Wilkes *et al.*, 2003). These pathways have not been well characterized with respect to TGF-β signalling, and have an unknown function in TGF-β biology. Of particular interest is the PI3K/Akt pathway, as it has been shown to be involved in both cell cycle and cellular growth (Brazil *et al.*, 2004). I wished to investigate the effects of TGF-β signalling upon the downstream target of PI3K, Akt and also study two direct targets of the protein kinase Akt, Foxo and GSK-3β differences in response both in cell culture and *Drosophila melanogaster.*

Chapter 1.2 TGF-β Superfamily

The TGF-β super-family of growth factors is found in all metazoans (Mehra and Wrana, 2002). They are grouped into 3 families: bone morphogenetic proteins (BMPs), Activins and TGF-βs. In addition, there are also several other cytokines that are more distantly related such as the Growth and Differentiation factors (GDF), Mullerian Inhibitory Substances (MIS) and Glial cell-line Derived Neurotrophic Factors (GDNF). The prototype of this superfamily is TGF-β1, a secreted peptide hormone that binds to a specific receptor and was first discovered in conditioned media of tissue culture where it confers a "transformed" phenotype on normal rat kidney fibroblasts in soft agar (NRK-49F) and an increase in the rate of collagen production and cellular growth (Assoian, 1985;Roberts *et al.*, 1986). These results implicated TGF-β as an important mediator of

tissue repair and transformation, hence the name. From the discovery of this growth factor, some 28 others have been identified, all homologous in key structural domains (Mehra *et al.*, 2002). Most of this superfamily are homodimers and have a cysteine knot in the protein structure as common features. They are secreted from many cell types as precursor molecules that are latent and are stored in the extracellular matrix (Hyytainen *et al.*, 2004). TGF-β is targeted to the ECM by latent TGF-β binding proteins (LTBP) to wait for the appropriate signal from the matrix then the ligands are cleaved internally to form the functional ligand that can act in an autocrine or paracine fashion. There are four LTBPs that seem to be specific for each of the three TGF-β ligands, and are present in different tissues, and have developmental differently spliced isoforms. Overall, LTBP may confer specify on highly similar ligands as they are divergent in sequence similarity and have unique expression patterns.

There are many varied functions of this superfamily, specific to the stage of development and the cell type upon which the ligand acts. For example, TGF-β causes many different cell type specific effects, such as being mitogenic to fibroblasts, growth inhibitory to epithelial/endothelial cells, a chemoattractant to macrophages and linked to both tumourgenesis and wound repair, neovascularization and differentiation of certain cell types (Massague, 1998). The Bone Morphogenetic Protein (BMPs) and Activin families have been associated more closely to the development of all metazoans. For example, Activins have an endocrine function in mammals and are involved in early development. As the name suggests, BMPs were first identified as factors that can induce mesodermal tissues (such as muscle) into bone but also function in patterning and

morphogenesis of early mesoderm in early development, neurogenesis, induction of the neural fate and induce somite formation (Massague, 1998). BMPs also act in the limb bud by reducing expansion and formation of the chondrocyte and osteoclast precursors (Kishigami *et al.*, 2005). After birth, they are involved in maintenance of bone mass, as demonstrated by conditional BMP 2/4 double knockout mice, which display slowed skeletal growth and postnatal bone formation. The Type II receptor for BMP2/4, bmpr1b also displays skeletal abnormalities. Dpp, the Drosophila BMP homolog is involved in dorsoventral patterning and specification of cell type in the early embryo.

Another TGF-β subfamily member, Activin is also involved with neurobiology. Activin family members are produced by neurons and are neuroprotective both *in vitro* and *in vivo* (Werner and Alzhimer, 2006). Activins are also involved in mesoderm formation, somatic growth, cell proliferation, apoptosis, branching morphogenesis inflammation and reproduction. Drosophila Activin is involved in neural wiring and proliferation, regulates mushroom body remodeling in the developing brain and motor neuron guidance in the embryo (Parker *et al.*, 2006). The Type I Activin Receptor, Baboon causes reduced proliferation within the optic lobe and central brain reduction in the size of the photoreceptor field (Brummel *et al.*, 1999, Zhu *et al.*, 2008)

Each of the TGF-β superfamily groups has unique receptors to carry out their diverse functions in the cell. These receptors, and their corresponding intracellular ligand are outlined in Table 1. While these receptor combinations are not exclusive, they are some of the most commonly found in these TGF-β subfamilies (Schmierer and Hill, 2007).

Table 1: Overview of the major TGF-β subfamilies

Adapted from Schmierer and Hill, 2007. Drosophila gene names are highlighted in blue.

Ligand "Family"	Ligands	Type II/I Receptors	Smads
TGF-β	TGF-β 1,2,3	TGF-βRII/ALK-5	Smad 2/3, SMOX
Activin	Activin βA,B,C,E dActivin, Dawdle	ActRII, ActRIIb, Punt / ALK 2, 4, 7, Baboon	Smad 2, 3, SMOX
BMP-2/4	BMP 2/4, DPP	BMPRII, Punt/ ALK 3,6 TKV	Smad 1,5,8, MAD
OP-1	BMP5, 6, 7, 8	BMPRII, Punt/ ALK 2,6 GBB, SCW	Smad 1,5, MAD
GDF-5	GDF 5, 6, 7	BMPRII / ALK-6	Smad 1,5,8 MAD

Chapter 1.2.1 Signalling of the TGF-β receptors

There are three types of TGF-β receptors called the Type I, II and III (TGF- βR1, II and III, respectively). Type I and II are the main 'signalling' receptors, with the type III receptor (B-glycan) being involved in some of the TGF-β functions such as presenting TGF-β2 ligand to the receptor complex and acting as a reservoir of active TGF-β (Massague, 2000). All TGF-β receptors classified to date, with the exception of type III, have serine-threonine kinase activity. The TGF- βRII receptor binds directly to a free ligand. It is a glycoprotein capable of autophosphorylation and constitutively active kinase activity, with a single extracellular domain and a trans-membrane spanning domain. The TGF-βI receptor (ALK 1-5, TGF-βRI being ALK5), has four main domains: extracellular ligand binding, transmembrane, a cytoplasmic juxtamembrane region containing a serine-glycine repeat region or the "GS" domain, and a C-terminus Ser-Thr kinase domain. The GS domain is a particularly interesting feature of this protein, being highly conserved among ALK receptors and trans phosphorylation of the region by TGF-βRII results in activation of the TGF-βRI kinase.

Together, TGF-βRI and RII form the functional signalling complex, as outlined in Figure 1. Ligand binds to the previously dimerized TGF-βII receptor (Henis *et al.*, 1994). Bound ligand/TGF-βRII recruits the 2 TGF-βI receptors into the complex, allowing the TGF-βII receptor to phosphorylate the TGF-βI receptors. Mutation of the GS domain (threonine 204 to aspartate) causes a constitutively active form of the TGF-βI receptor in the absence of ligand binding. This creates a charged amino acid substitution

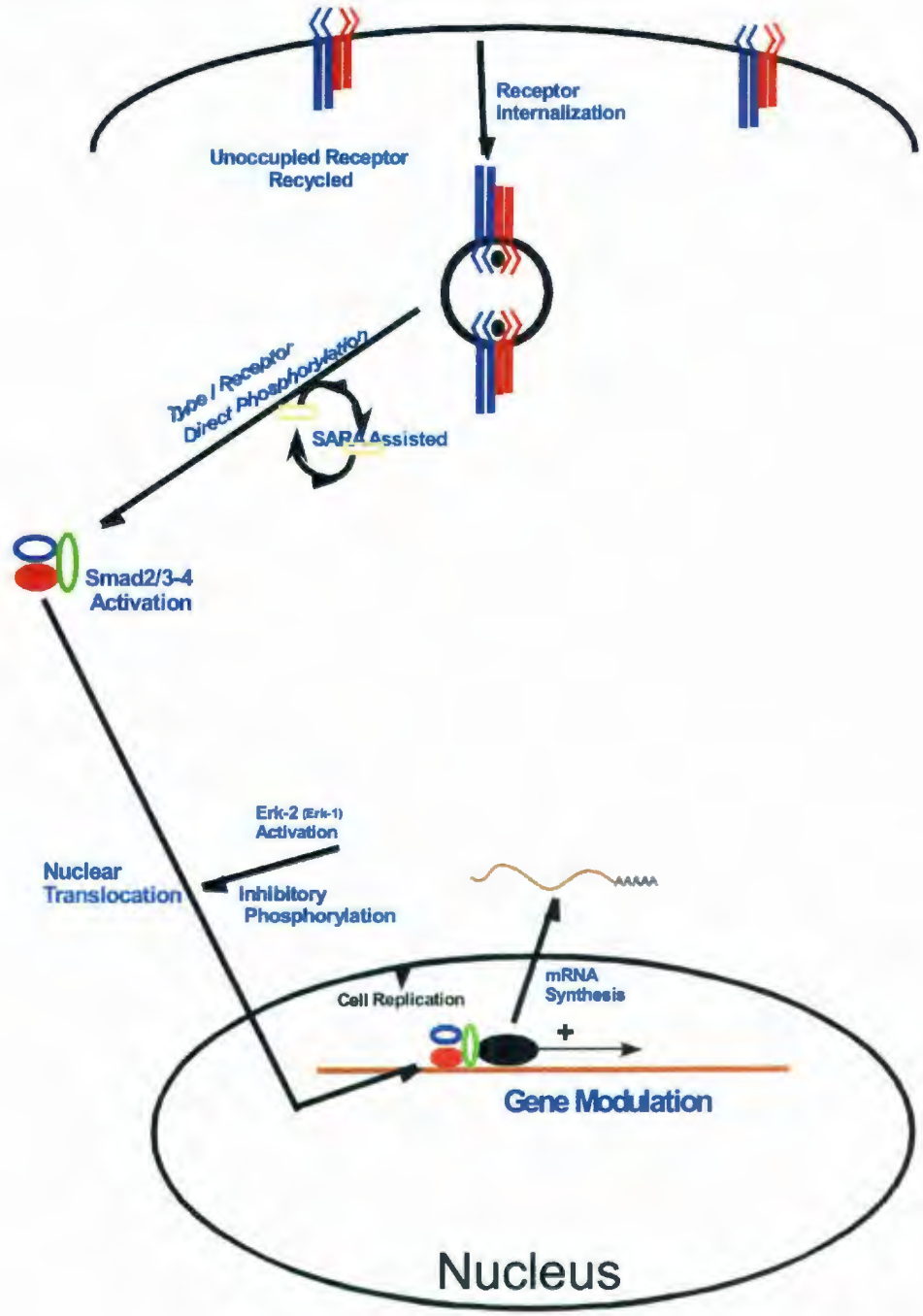

Figure 1: Overview of known TGF-β Signalling Pathway

TGF-β receptors are endocytosed into the cell after ligand binding. This leads to the Type 2 receptor phosphorylation of the Type I receptor, resultant in kinase activity. Type I receptors can then phosphorylate R-Smads 2/3, which are brought to the receptor by Smad anchor for receptor activation (SARA). Smad 2/3 then binds with the co-Smad4 to undergo nuclear translocation, which ultimately leads to gene transcription or repression. exist as a dimer), within the GS domain region (Wrana *et al.*, 1994).

that confers constitutive activity in the absence of ligand (Wieser and Massague, 1995).These amino acid substitution does not seem to be a site that is phosphorylated in Type I receptor activation, but instead lies just outside the site. This 'constitutive activity' is also independent of Type II Receptor binding, and is not conserved amongst all TGF-β like receptors, suggesting this may be unique to the TGF-βRI receptor. This activated receptor seems to have greater activity than wild-type Type I receptors, which indicates that it may not directly mimic natural activation of TGF-β signaling. Once the TGF-βI receptor is 'activated' it phosphorylates receptor Smads (R-Smads) at specific serine residues (Nakao *et al.*, 1997). The activation of the signaling complex is still an open question, with some evidence being presented that receptor endocytosis being needed for complete activation. This is thought to occur by bringing all the TGF-β signaling components together in the same compartment. The FYVE domain protein SARA promotes uptake of the SARA/Smad complex to endosomal membranes promoting clathrin-dependent endocytosis and receptor-mediated activation of Smad proteins (Caestecker *et al.*, 2004). There are also sorting nexins which interact with Type I receptors and help traffic them across the plasma membrane. A protein called Hgs helps traffic Smad 2 and 3 to the Activin Type I receptor, allowing for different receptors to have specificity. This is important, as it as been demonstrated in many systems such as Xenopus the distribution of Smad-2 is dynamic (Lee *et al.*, 2004). Extracellular antagonists in frog embryos causes nuclear phosphorylated Smad2 to disappear. This suggests that constitutive cycling of the receptor, and that they are cycled back and forth the cell membrane in the absence of ligand. However, there is also shunting to other

endocytic compartments, which argues against the theory that this is a 'signalling event'. There is evidence that endocytosis of the receptor has a role in dampening receptor dependent signaling though degradation. This also would also explain how nuclear Smad-2 can 'disappear' very quickly in the early Xenopus embryo (Lee *et al.*, 2004). Therefore, where within the cell the signal originates can confer additional specificity to the signalling process.

The main known signal transducers of TGF-β signalling are Smads (Massague, 2000). There are three different subgroups of Smads: the receptor Smads, the Co-Smads and the inhibitory Smads. The receptor Smads are specific to the ligand/receptor complex, with Smad 1, 5 and 8 associating with the BMP receptors, and Smad 2 and 3 downstream of TGF-β/Activin receptors. R-Smads are phosphorylated by the Type I receptor on a conserved carboxy-terminal SSXS motif. They then dissociate from the receptor and form a heteromeric complex with the co-Smad4 to allow nuclear translocation. Once in the nucleus, the Smad complex can bind with various partners to repress or activate gene transcription. However, the story is far more complex than originally thought when the Smad pathway was first identified in Drosophila and *C. elegans* (Sekelsky *et al.*, 1995;Raftery *et al.*, 1995;Savage *et al.*, 1996). One classical example of how TGF-β signals confer specificity is the L45 loop in the TGF-β Type I receptors that allow them to recognize the appropriate Smads (Persson *et al.*, 1998). This loop restrict Smads 2/3 to TGF-β/Activin receptors with only one key amino acid being different from the BMP Type I receptors. The R-Smads themselves also have a region to confer specificity, the L3 loop that is 17 amino acids long in the C-terminal domain (Lo

et al., 1998). If the different amino acids are 'swapped' between Smad1 and Smad2, they each will respond to the other 'normal' type I receptor (Smad1 to TGF-β, Smad2 to the BMP receptors).

In addition to the promotion of TGF-β signalling, one mode of regulation of the signaling pathway utilizes inhibitory Smads (Smad 7) to decrease TGF-β signalling (Hayashi *et al.*, 1997). Inhibitory Smads are turned on by TGF-β signalling itself through transcription via R-Smads. This acts as a 'fine-tuning' mechanism for signalling and allows signalling to be turned off after the appropriate amount of time has passed. I-Smads are thought to act via 1) direct interaction with Type I receptors to compete with R-Smad binding and 2) targeting receptor complexes for destruction (Schmierer and Hill, 2007). This event requires ubiquitin ligases, Smad ubiqutination regulatory factors 1 & 2 (SMURF 1 and 2) to tag the activated Type I receptor complex and the R-Smads for degradation (Kavsak, 2000). Smad-7 can also interact with PP-1 to target the receptor complexes (Schmierer and Hill, 2007). A nuclear role for Smad-7 has been suggested, as Smad-7 may be able to bind Smad-responsive elements. Therefore, Smad-7 may also be able to interfere with already active Smad complexes by competing for binding to Smad-responsive elements.

Besides the Smad-dependent pathways, a number of Smad-independent pathways have been noted. TGF-β has been shown to regulate MAPK signalling, both independently and through RAS, ERK, Jun and Wnt signaling (Derynck and Zhang, 2003). As well, TGF-β has recently been shown to activate PI3K in mesenchymal cell types (Wilkes *et al.*, 2005) and the downstream targets p21 Activated Kinase-2 (PAK2)

and Akt. However, there is controversy over this pathway with different groups getting different results, reporting activation of Akt (Ju *et al.*, 2005), while others see none, even within the same cell type (Wilkes *et al.*, 2005).

TGF-β signaling is complex, with Smad signalling being the only well-characterized pathway. However, as the Smad signal transduction pathway appears to be the same in all cell types, in an effort to distinguish fibroblast and epithelial cell responses the rest of this review will focus on Smad independent signaling.

Chapter 1.2.2 Smad Independent TGF-β Signalling

Several Smad independent pathways have been observed. Ras has been implicated in TGF-β biology, with constitutively active forms of Ras interacting with TGF-β signaling, and TGF-β directly activating Ras in epithelial cells (Yue and Mulder, 2000;Janda *et al.*, 2002). Other pathways that have been implicated in TGF-β signaling are extracellular regulated kinase (ERK), JNK and p38 MAPK pathways (Mulder, 2000;Yu *et al.*, 2002;Derynck *et al.*, 2003;Kim *et al.*, 2004). TGF-β can activate p38 MAPK in certain epithelial cultures (Yu *et al.*, 2002). In systems with mutations in the TGF-β Type I receptor that cannot activate Smads, TGF-β still activate p38 MAPK. This Type I receptor is mutant in the L45 loop, so it should be activating p38 MAPK independently of Smads. Use of dominant negative Smads also does not interfere with the activation of p38 MAPK indicating this is a Smad-independent event. However, the role that these two pathways play in TGF-β biology is poorly understood. Both ERK and JNK pathways, downstream of Ras, have been shown to phosphorylate Smads at specific sites.

These phosphorylation sites cause decreased transcriptional activity and cytoplasmic relocalization of the Smads when phosphorylated. ERK and p38 MAPK have been linked to epithelial-mesynchemal transition (EMT, Bhowmick *et al.*, 2001;Yu *et al.*, 2002), however vastly different epithelial cell lines were used in these reports and similar results have not been replicated in later work. Some of these reports also rely upon pharmacological inhibitors of p38 MAPK that can also inhibit the TGF-β receptor kinases, at doses higher than 5 μM (Fu *et al.*, 2003). As well, it is not clear that these events are truly Smad-independent as one group does report that Smads are required for EMT (Yu *et al.*, 2002).

Along with ERK, TGF-β activates the Rho-like GTPases, Rho A, Rac1 and cdc42 (Edlund, 2002). Typically, the activation of these proteins has been linked to PI3K and cytoskeletal reorganization downstream of receptor tyrosine kinases (RTKs). When activated by TGF-β, they have been linked to cytoskeletal reorganization and EMT. There has also been a report that the LIM1 kinase can interact with the cytoplasmic tail of the BMP Type II receptor, and this receptor can phosphorylate LIMK1 directly (Foletta *et al.*, 2003). LIMK1 kinases regulate the small G protein family, Rho, and are involved in actin cytoskeleton reorganization. The small GTP'ase Cdc42 is required for activation of LIMK1 after BMP stimulation. LIMK1 can then translocate to the nucleus and represses Rac/CDC42 dependent Cyclin D1 expression. Experiments in Drosophila with Dpp also indicate that DCDC-42 and dPAK are activated in response to Dpp signaling, but LIMK1 may or may not be involved (Ricos *et al.*, 1999). A key question is whenever the c-

terminal tails of other Type II receptors can signal with LIMK1, and is an open question with regards to TGF-β biology.

A potential downstream target of Rac1/cdc42, is the p21-activated kinase (PAK2). TGF-β has been shown to specifically activate PAK2 in a subset of fibroblast cell lines, but not epithelial cells in a pathway that requires cdc42 (Wilkes *et al.*, 2003). This event was shown to be independent of Smad signaling and dependent on TGF-β receptors. Abolishment of PAK2 leads to defects in TGF-β mediated growth and morphological transformation (MT) in these fibroblast cultures. A more recent report (Wilkes *et al.*, 2005) has linked TGF-β to both PI3K and PAK2 in a linear pathway, giving a clear signaling mechanism of how TGF-β exerts its action upon PAK2.

A link between TGF-β and PI3K signaling pathways in invertebrates was first established in 1997, with the discovery that there was a genetic interaction in *C. elegans* between Daf-7 (a TGF-β ligand homologue) and Daf-16 (FOXO homologue, Ogg *et al.*, 1997). A role for PI3K has also been described in EMT of epithelial cultures downstream of TGF-β and in preventing apoptosis of epithelial cultures through activation of the PI3K target, Akt (Bakin *et al.*, 2000;Shin *et al.*, 2001). One of the major issues with Smad-independent signalling is exactly how TGF-β receptors are able to couple to PI3K. PI3 Kinases are traditionally activated by receptor tyrosine kinases, and TGF-β's are serine/threonine kinases. TGF-β has been linked to PI3K activation indirectly in well over 300 papers and counting in Pubmed, but there are only a few reports that address the issue. Yi *et al.* (2005) immunoprecipitated the PI3K p85 subunit from TGF-β treated cells and found that this subunit could increase PIP3, pAkt and pErk after 1 hour of

stimulation. When COS-7 fibroid cells were transfected with p85 subunit and TGF-β Type I and II receptors, the authors were able to always pull-down p85 with the Type II receptor, and only in the presence of ligand in the case of the Type I receptor. To assess whenever this was a direct interaction, *in vitro* assays with p85 and the Type I and II receptors were undertaken. In this assay, only the Type II receptor could bind p85, suggesting that the Type I receptor requires some cofactors or adaptor proteins to bind PI3K. Kinase deficient Type II receptor disrupts endogenous binding of PI3K to the Type I receptor, and the Type I receptor kinase activity seems to be essential for PI3K kinase activity, as assayed by pAkt.

These findings are somewhat supported by a report by Wilkes *et al.* (2005) which demonstrates that TGF-β activates PI3K in mesencyhmal cultures. In fibroblasts, p85 was immunoprecipated from TGF-β treated cells and resulted in increased kinase activity against PIP. Activation of pAkt at Serine 473 and Theronine 308 was also observed. The findings of Yi were also extended in that inhibitors of transcription and translation didn't prevent Akt phosphosrylation, suggesting this is a transcription factor independent event. Knockdown of p85 by morpholino also prevented Akt phosphorylation in the fibroblast cells. However, in the Wilkes paper, there was no activation of PI3K seen in epithelial cells, which is in conflict with the Yi report.

This increased activation of PI3K has been shown in fibroblastic cell lineages as well as epithelial cultures as demonstrated by the above reports (Kim *et al.*, 2002). However, especially with epithelial cultures, several groups fail to show "activation" of Akt when stimulated with TGF-β in epithelial cells (Conery *et al.*, 2004;Seoane *et al.*,

2004;Wilkes *et al.*, 2005). To clarify this issue, one of the major goals of this thesis will be to further define the interaction of TGF-β with Akt and some of it's downstream targets.

Chapter 1.3 PI3K Signalling Pathways

Many extracellular signals control the delicate balance between cell survival and cell death (Brazil and Hemmings, 2001). These signals can function by activating phosphatidylinositol 3-OH kinase (PI3K), an enzyme that catalyzes the addition of a phosphate group to the inositol group of phospholipids. One of the first ligands shown in activation of PI3K was Insulin. However, PI3K has been shown to be activated by several growth factors other than Insulin (Lawlor and Alessi, 2001) such as Epidermal Growth Factor (EGF), Insulin-like Growth Factors (IGF-1, 2), Nerve Growth Factor (NGF), Vascular Endothelial Growth Factor and Platelet Derived Growth Factor (PDGF, Brazil *et al.*, 2001). All of these growth factors have been implicated in human health and disease, with EGF (Her-2/Neu) being particularly relevant to female specific cancers, such as breast and ovarian cancer. In Drosophila, PI3K has been shown to be activated by the insulin-like peptides (DILPs, Brogiolo *et al.*, 2001). All of these ligands have known receptors, such as the EGF receptor and the insulin receptor. Most of the receptors known to activate PI3K are RTKs, although some G-protein coupled receptors such as the endothelin receptor can also activate PI3K (Brazil *et al.*, 2001). RTKs once activated, dimerize, and undergo an auto-transphosphorylation event at a number of tyrosine residues. Through these phosphotyrosines, downstream proteins recognize the activated receptor. The p85 regulatory subunits of PI3K (p60 in Drosophila) have an Src homology

2 domain (SH2) that recognizes these phosphorylated tyrosines. However, the affinity of p85 for RTKs are helped by a class of molecules called adaptor proteins (Zhang and Samelson, 2000). Adaptor proteins are proteins that do not contain motifs for transcriptional activity or kinase activity. Instead, they are made of motifs designed to recognize phospho-tyrosine YxxM motifs, such as SH2 and SH3 or phospho-tyrosine binding (PTB) domains. Some examples of adaptor proteins that help PI3K bind to EGFR are Gab, Sos and Grb. For the insulin receptor, PI3K activation is enhanced by the adaptor protein, insulin receptor substrate 1-4 (IRS1-4, Folli *et al.*, 1992;Lawlor *et al.*, 2001). Once bound either directly or by the help of adaptor proteins, p85 recruits and activates the catalytic p110 subunit (Vanhaesebroeck *et al.*, 1997). The removal of these growth factors from culture medium results in the inactivation of PI3K, thus apoptosis or cell cycle arrest due to the cell survival pathway of PI3K/Akt being no longer active.

Activation of PI3K by the relevant RTK leads to the subsequent activation of Akt and other kinases through membrane lipid phosphorylation (Leevers *et al.*, 1996;Chan *et al.*, 1999). There are three classes of PI3 Kinases, the Class 1A/B, Class 2 and Class 3. PI3Ks are classified based on which PtdIns they phosphorylate *in vitro*, with class IA PI3Ks also showing a preference for PtdIns(4,5) bisphosphate , *in vivo* (Vanhaesebroeck *et al.*, 1997). Since only the PI3K1A kinases activate Akt, these will be further discussed. PI31A kinases are lipid/protein kinases composed of heterodimers which consist of a regulatory subunit (p85) and a catalytic subunit (p110) (Vanhaesebroeck *et al.*, 1997). The p85 subunit SH2 domain binds to the phosphorylated tyrosines of RTKs in the plasma membrane. This binding localizes the PI3K from the cytosol to the membrane, the

location of the kinase's lipid substrates. The assembled kinase then phosphorylates the D-3 position on the ring of inositol lipids to generate phosphatidylinositol 3-phosphate (PIP), phosphatidylinositol (3,4) bisphosphate (PIP2) and phosphatidylinositol (3,4,5) triphosphate (PIP3) from PtdIns, PtdIns(4)P and PtdIns(4,5)P2 respectively. The phosphorylated lipids can then be bound by pleckstrin homology (PH) domains on various downstream proteins (Chan *et al.*, 1999) such as cdc42/Rac1, Phosphoinositide-dependent kinase 1 (PDK-1), Intergrin Linked Kinase (ILK) and Akt.

Chapter 1.3.1 Akt

Akt is a serine/threonine kinase that is activated by the activity of PI3K (Datta *et al.*, 1996;Lawlor *et al.*, 2001). The Akt protein contains a PH domain at its N-terminus, a glycine rich domain and a serine/threonine kinase domain. It also contains two regulatory phosphorylation sites, located at threonine 308 and Serine 473 of Akt1, with similar sites in Akt2 and Akt3 (Brazil *et al.*, 2004). *Drosophila melanogaster* has one form of Akt (Dakt) (Staveley *et al.*, 1998), while mammals have 3 isoforms (Akt 1, 2, and 3). Akt is activated by phosphorylation at the key residues, $Akt1^{Thr308}$ and $Akt1^{Ser473}$ as illustrated in Figure 2 (Brazil *et al.*, 2004). It is phosphorylated by PDK1 at $Akt1^{Thr308}$ (Rintelen *et al.*, 2001).

The identity of the kinase that has Phosphoinositide-dependent kinase-2 (PDK-2) function remains controversial (Chan and Tsichlis, 2001). A number of kinases may phosphorylate at $Akt1^{Ser473}$, such as autophosphorylation by Akt itself, but this PDK2 function is elusive, as none has been shown conclusively *in vivo* (Chan *et al.*, 2001).

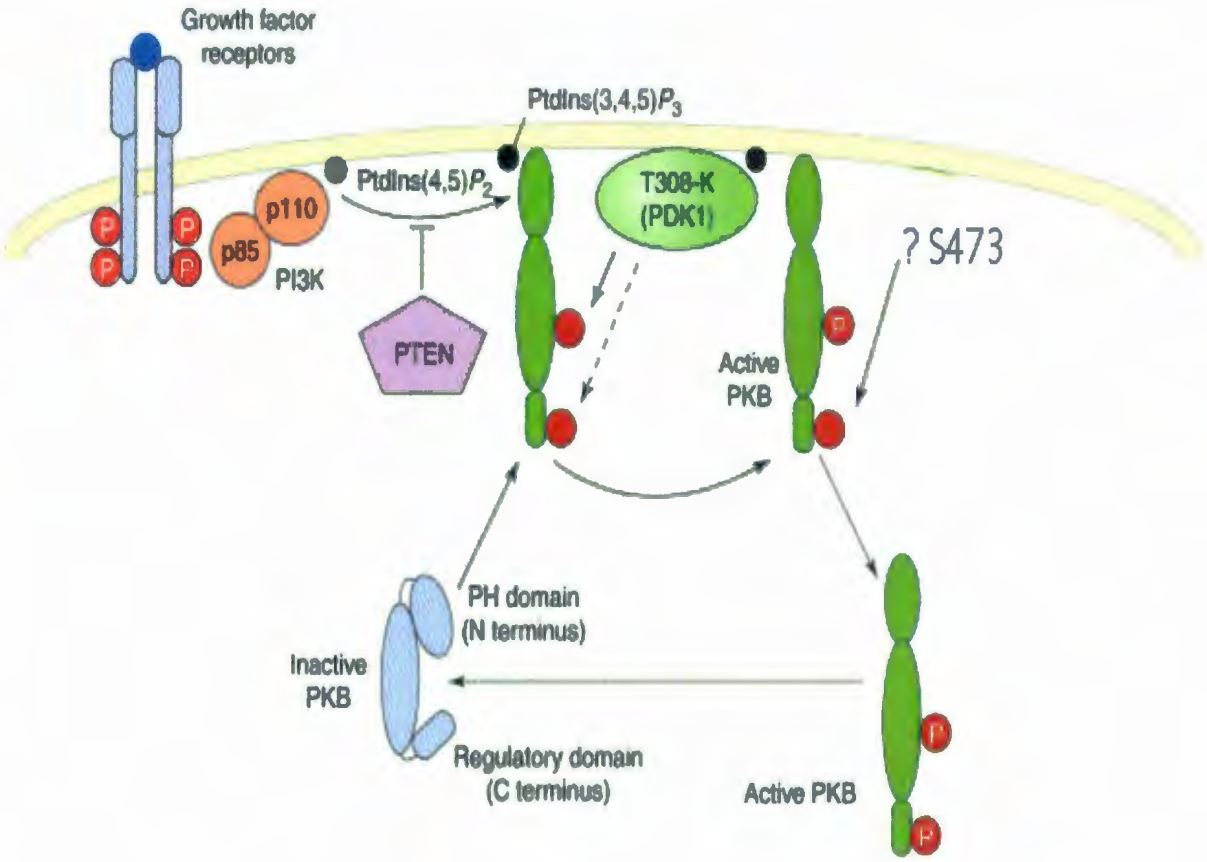

Figure 2: An overview of Akt activation by PI3K.
Growth factors bind to receptors, and recruit PI3K to the cell membrane. PI3K phosphorylates PtdIns(4,5)P_2 give PtdIns(3,4,5)P_3. This recruits PDK1 and Akt to the membrane, where PDK1 phosophorylates residue Thr 308. An unknown kinase phosphorylates Ser 473. Once activated, Akt dissociates for the cell membrane to phosphorylate substrates (FOXO) in the cytosol and nucleus. This figure was adapted from Brazil and Hemmings, 2001.

An essential role for Intergrin Linked Kinase (ILK) in Serine 473 phosphorylation has been demonstrated by RNA interference, however fibroblast from ILK-knockout mice do not appear to have reduced Serine 473 phosphorylation when compared to wild-type (Attwell, 2003; Shioi *et al.*, 2002). Target of rapamycin (TOR) kinase and its associated protein rictor is another potential Serine 473 kinase (Sartbassov *et al.*, 2005) in cell culture, however this has not been demonstrated *in vivo.* As the controversy suggests, there role of PDK-2 may be assumed by a number of kinases depending on the cellular and developmental context.

PDK1 is activated by the presence of PtdIns (3, 4, 5) P3 produced by the action of PI3K. Binding of the lipid to PDK1's PH domain, causes recruitment to the cell membrane (Rintelen *et al.*, 2001), resulting in a conformational change that activates PDK1. Phosphorylation on the activation loop site $Serine^{241}$ is also necessary for activation of PDK-1 and appears to be an autoactivator event (Casamayor *et al.,* 1999). As well, PIP3 interacts with the PH domain of Akt, to colocalize Akt and PDK1 to the plasma membrane. Akt is phosphorylated within its p-loop domain containing Thr^{308} by PDK. This activation process following ligand binding can be quite rapid, and activation of Akt occurs maximally within a few minutes, as in the cause of EGF (Burgering and Coffer, 1995). This contrasts with growth factors such as insulin, that maximally induces Akt phosphorylation after about 90 to 120 minutes after ligand stimulation (Ueki *et al.*, 1998), suggesting multiple ways of activating Akt through the common mechanism of PI3K activation.

Chapter 1.3.3 Downstream targets of Akt

Once activation occurs, Akt dissociates from the plasma membrane and moves into both the cytoplasm and nucleus where it can act upon its substrates (Burgering and Medema, 2003). Many of the effects of Akt on cell cycle may be through the inactivation of components of cell cycle inhibitory pathways, such as $p21^{Cip1}$ and $p27^{Kip1}$ (Liang *et al.*, 2002). The effects of Akt on cell survival may be through the inactivation of components of apoptotic pathways, such as the phosphorylation of Bcl-2 pro-apoptotic family member (BAD, Datta *et al.*, 1997). The phosphorylation of BAD results in its binding to 14-3-3 protein. This prevents BAD from interacting with Bcl-xL, thus preventing induction of apoptosis. In addition, Akt phosphorylates the inhibitor of NFκB kinase-α (IKKα, Brazil *et al.*, 2001). As the name suggests, IKKα inhibits nuclear factor κB (NFκB) under normal circumstances. When phosphorylated by Akt, IKK is inactivated, allowing NFκB to act as a transcription factor for induction of cell survival genes.

A very important target of Akt activity is glycogen synthase kinase-3 (GSK-3). originally identified as a protein involved in glucose metabolism (Doble and Woodgett, 2003). This protein is a serine/threonine kinase with many diverse substrates in the cell. There are two isoforms of GSK-3, α and β, in mammals. Both isoforms are similar in the kinase domain, however different in other parts of the protein. There is one isoform in Drosophila termed Shaggy or Zeste-White 3 (Park *et al.*, 1998). The two mammalian isoforms appear to phosphorylate similar substrates. However, the generation of GSK-knock-out mice demonstrates these proteins are not redundant. The GSK-3β knockout was embryonic lethal due to massive apoptosis of liver cells. This defect could not be

rescued by the overexpression of GSK-3α (Hoeflich *et al.*, 2000). As most of the literature has described GSK-3β substrates, we chose to focus on this isoform.

GSK-3β has several phosphorylation sites that are involved in the regulation of the protein. This includes one at the c-terminus (T-loop), GSK-3β^{Tyr216}, that is constitutively phosphorylated and thought to be necessary for the activation of the protein. Interestingly, this site has been shown to be necessary for substrate phosphorylation, but does not affect kinase activity (Dajani *et al.*, 2001). Since GSK3 shows a strong preference for 'primed' substrates, ones that have already been phosphorylated upstream of the GSK3 recognition sites, perhaps this site is necessary for the recognition of these substrates. Variations of phosphorylation at this site have been noted in different cell types (Papadopoulou *et al.*, 2004), and knocking this site out in Drosophila eliminates function of the protein. However, the main mechanism of GSK-3β regulation is the phosphorylation of the serine residue located at position 9. Phosphorylation at GSK-3β^{Ser9} is an inhibitory event, preventing kinase activity by preventing the protein from binding substrate (Cross *et al.*, 1995), and this is the site that is phosphorylated by Akt.

Active GSK-3β has numerous substrates that are involved in gene transcription. Phosphorylation of these substrates results in their inactivation, thus regulating cellular events such as growth, protein translation, cell cycle and apoptosis. In addition to regulation by direct phosphorylation by Akt, GSK-3β is functionally regulated by Wnt signalling (Patel *et al.*, 2004). After Wnt ligand binding, the protein Dishevelled causes GSK3β to be inactivated through an unclear mechanism, perhaps involving the APC complex (van Noort *et al.*, 2002a). This places GSK3β downstream of both PI3K and

Wnt signalling, allowing for potential cross-talk between the two pathways. However, at least in some cell types, insulin inhibits GSK3β activity but has no effect on Wnt signalling indicating the two pathways intersecting at GSK3β may be functionally distinct (Doble *et al.*, 2003). One of the best-characterized components of Wnt signalling is β–Catenin, a tumour suppressor that can induce transcription of Cyclin D1. GSK-3β can phosphorylate β-Catenin leading to proteosomal mediated degradation of the protein (van Noort *et al.*, 2002b). However, the impact of Akt in affecting β-Catenin appears to depend on cell type and context. GSK-3β can also phosphorylate eIF2B, as well as c-myc. These transcription factors upregulate the expression of many cell cycle proteins, notably Cyclin D1 (Doble *et al.*, 2003). In addition to these effects on Cyclin D1 transcription, GSK-3β has been shown to directly modulate Cyclin D1 by phosphorylation at a threonine located at site 286 (Diehl *et al.*, 1998). This phosphorylation in NIH-3T3 cells was shown to decrease the stability of Cyclin D1 (increasing the protein turnover rate by 50%), complex formation with CDK-4/6 and the nuclear localization of Cyclin D1. An interesting finding was that GSK-3β recognizes Cyclin D1 best as a substrate when it is complexed with its CDK binding partner. This suggests that GSK-3β has a direct effect on cell cycle progression.

GSK-3β also has effects on Tau and Presenilin 1, two proteins known to be involved in Alzheimer's disease pathology (Doble *et al.*, 2003). The Drosophila homologue, Shaggy, has been shown to hyper-phosphorylate tau and induce neurofibrillary tangles in a Drosophila model of Alzheimer's disease, consistent with the notion that hypo-phosphorylation of Tau leads to some of the disease state phenotypes (Jackson *et al.*, 2002). To date, the only well-characterized substrates of Shaggy are Tau

and β-Catenin, and these have been studied strictly with regard to Wingless/Wnt signaling (Ruel *et al.*, 1999). It has been postulated that Shaggy acts downstream of dPI3K/dAkt, in a similar fashion to mammals (Staveley, personal communication), however no group has shown this to date.

Another disease state that GSK-3β has been implicated in is cancer development, through its regulation of the previously described substrates, and more recently Snail. The Snail transcription factor was originally identified in Drosophila as an EMT transducer, and has since been implicated in human cancer (Peinado *et al.*, 2003). GSK-3β phosphorylates Snail at two sites, inducing nuclear relocalization and degradation of the protein to prevent EMT in epithelial cells. Interestingly, a report has demonstrated a link between Snail and TGF-β (Peinado *et al.*, 2003). Snail protein levels are increased upon TGF-β stimulation in epithelial cells and Snail has been implicated in TGF-β mediated EMT via an unknown mechanism.

An additional downstream target of Akt is the transcription factor, FOXO (forkhead box, sub-group "O") or FKHR (forkhead in rabdomyosarcoma), which are part of a larger group of transcription factors termed the Fox family (Brunet, 2004). There are four isoforms of FOXO in humans, FOXO1, 3a, 4 and 6 (van der Heide *et al.*, 2004) and one in Drosophila, dFOXO (Kramer *et al.*, 2003). FOXO is a member of the winged helix transcription factor family and contains a nuclear localization signal (NLS), a nuclear export signal (NES), Akt phosphorylation sites and a forkhead box DNA binding domain (Tran *et al.*, 2003). Akt can phosphorylate FOXO1 at three specific sites, $FOXO1^{thr24}$, $FOXO1^{ser256}$ and $FOXO1^{ser319}$. Upon phosphorylation, FOXO is then excluded from the nucleus and is sequestered in the cytoplasm by a 14-3-3 binding protein. If Akt is

inactive, then FOXO remains in the nucleus, where it induces transcription of a number of target genes.

FOXO causes the transcription of several factors that lead to control of cell cycle, such as $p27^{Kip1}$. As FOXO was originally identified as a tumour suppressor gene in human sarcoma, the regulation of these genes may be how loss of this protein promotes cancer. To facilitate G1 arrest, FOXO upregulates transcription of the Cyclin-Dependent Kinase Inhibitor $p27^{Kip1}$ (Burgering *et al.*, 2003) and inhibits transcription of other factors such as Cyclin D1 (Schmidt *et al.*, 2002). FOXO is also involved in the G2/M phase of the cell cycle via the transcription of GADD45 (Furukawa-Hibi *et al.*, 2002 ; Tran *et al.*, 2002). GADD45 is a DNA repair protein and FOXO may help stall the cell at G2/M after oxidative stress to allow DNA repair to occur. In addition to these effects on cell cycle, dFOXO has been shown to influence growth and nutrition in Drosophila (Giannakou *et al.*, 2004)

Overexpression of dFOXO induces smaller cell size and also decreases number of cells in the fly eye (Kramer *et al.* 2003). DAkt rescues the dFOXO smaller cell size phenotype, supporting the idea that DAkt regulates growth through dFOXO. Overexpression of the dFOXO phenotype is rescued by dRAS in the eye. However, no effect was seen with EGF, suggesting that dFOXO mediates growth through inhibition of cell cycle and not apoptosis. Akt, and its direct downstream target, FOXO, therefore function in the direct control of both growth regulation and cell cycle

Chapter 1.4 Cell Cycle Proteins

An important input into cell growth is the control of the cell cycle. In embryonic cell lineages, the cell cycle is short, with S and M phases being dominant with little time

spent in the 'Gap' phase (Sherr, 2000a). As cells differentiate into their final fates, the two Gap phases (G1 and G2) become crucial for cell cycle control. In the first 'Gap' phase, or G1, the cell increases size and "measures" nutrient availability to ensure sufficient resources to synthesize DNA and make a daughter cell. Therefore, this part of the cell cycle is controlled by many mitogenic signals or growth factors allowing the start of the cell cycle to be coupled to metabolic and outside inputs.

Indeed, many differentiated cells do not divide (e.g. neurons), entering a 'G_0' stage either temporary or indefinitely. In order for a cell to escape 'G_O' or senescence, some form of initiation factor must be applied. These initiation factors mainly cause the increased transcription/synthesis of the D type family of cyclins (Sherr, 2000b). Cyclins are proteins that by themselves have little activity, but when bound to Cyclin-Dependent Kinases (CDK) in a heterodimer, create an active protein kinase (Massague, 2004). The CDKs are regulated by phosphorylation, but it is mainly the protein interactions with the cyclins that determines functionally. The CDK binding partners for the D family of Cyclins are CDK-4 and 6. The prototype of the D family is Cyclin D1 that mainly binds to CDK-4. The major physiological substrate of this complex is the retinoblastoma protein (Rb, Zhang and Dean, 2001). Rb is a tumour suppressor that regulates the E2F transcription factors. Binding of Rb either turns the E2Fs into transcriptional repressor proteins (E2F4, 5) or inactive transactivators of transcription (e2F 1, 2, 3), thereby eliminating their respective functions (Zhang *et al.*, 1999). Transcription of the Cyclin E gene requires functional E2F transcriptional factors. Increased Cyclin E levels are needed to make functional Cyclin E-CDK2 complex. Therefore, Cyclin D1/CDK4 complexes function to inactivate Rb, thereby releasing E2F to induce Cyclin E and other genes

involved in DNA synthesis. Active Cyclin E-CDK2 complexes phosphorylate the remaining Rb, allowing for entry into S phase.

In this model of the cell cycle, a cell that has any stimulation with growth factors will replicate. This is not necessarily the case, since the cell can regulate the timing and control of cell division indicating many inputs into this process (Sherr, 2000b). It is known that the expression and formation of the Cyclin/CDK complexes are tightly regulated processes that occur only at a very specific time in the cell cycle. To increase the ability of the cell to regulate Cyclin/CDK activity, there are CDK-inhibitors that block complex formation. They are $p27^{Kip1}$, $p14^{ink4b}$, $p16^{INK4a}$ and $p21^{Cip1/Waf1}$. The main inhibitor of the cyclin E/CDK2 complexes is $p27^{Kip1}$ and forced expression of this protein can halt the cell cycle (Sherr and Roberts, 2004). However, there are several mechanisms by which mitogenic stimuli can inactivate p27, either at the transcriptional/translational level or by direct phosphorylation. Phosphorylation of p27 decreases stability of the protein and causes nuclear exclusion. Surprisingly, p27 has also been shown to be present in Cyclin D1/CDK complexes, and the presence of p27 appears to help complex formation (Polyak *et al.*, 1994;Reed, 2002). Therefore, Cyclin D1/CDK 4-6 complexes function in the cell cycle by mopping up any excess p27 so that Cyclin E/CDK2 can become active. Once this Cyclin E/CDK 2 complex is active, it will phosphorylate p27, ultimately driving the cell through the G1/S checkpoint.

Chapter 1.5 Experimental Rationale

Both TGF-β and PI3K/Akt pathways regulate the cell cycle (Liang *et al.*, 2002), with Akt, in addition to the previously mentioned targets, directly regulating the phosphorylation of $p27^{kip1}$ and $p21^{cip1}$, which causes degradation and nuclear exclusion of

these proteins (Fujita *et al.*, 2002). In a report by Seaone *et al.*, (2004), Smad 2/3 was shown to cooperate with FOXO transcription factors to induce $p21^{cip1}$ gene expression in cells of epithelial origin. This report links the TGF-β and PI3K signalling pathways together in the control of a cell cycle gene. In Drosophila, nutritional signals can activate quiescent larval tissues to enter the cell cycle and continue larval growth (Britton and Edgar, 1998). As well, ectopically expressing genes known to contribute to the cell cycle can stimulate growth. Mutations in the insulin signalling components chico, dInR , Dp110, PDK1, Akt and dS6K results in smaller flies (Leevers *et al.*, 1996;Goberdhan *et al.*, 1999;Verdu *et al.*, 1999;Rintelen *et al.*, 2001). Overexpression of Dp110 (dPI3K) increases cell size, but not cell division rates (Leevers *et al.*, 1996). Work by Staveley *et al.*, (1998) illustrates that mutations in *DAkt* cause ectopic apoptosis and smaller body size. In parallel to the insulin pathway, mutations in Baboon, the TGF-β/Activin TGF-βI receptor, result in reduced size of nervous system imaginal discs and overall organ size, without increasing apoptosis (Brummel *et al.*, 1999) This suggests that the effects of PI3K/Akt and TGF-β are linked in the control of cell cycle, as this is an integral part of cellular growth.

A major goal of this research is to investigate the molecular signalling pathways and physiological roles of TGF-β. As outlined above, TGF- β has been shown to activate SMADs to exert its effects and also interacts with PI3K pathways via an unknown mechanism. One of the goals of this project is to further characterize the interaction of TGF- β signalling with Akt. We will also study two direct targets of the protein kinase Akt, FOXO and GSK-3β.

A major theme of this research is to determine why different cell types respond in very dissimilar fashion to TGF-β. Epithelial and immune cells are growth inhibited by TGF- β, whereas mesenchymal cells are growth simulated (Massague, 2000). Prior to this research, both categories of cells appear to activate identical receptors and the same downstream intracellular pathway (SMADs). Therefore, I am looking for differences in the PI3K pathway between fibroblast (mesenchymal cells) and epithelial cells.

In addition to the studies that will be undertaken in cell culture, I will also try to determine a genetic interaction in *Drosophila melanogaster*. Since overexpression/null alleles of many PI3K pathway components and TGF-β receptors cause viability problems in whole flies, I will study the interactions in the compound eye. The Drosophila compound eye is an excellent model for studying cell growth/developmental genes due to its highly organized structure of ommatidia. Using UAS/GAL4 system of ectopic gene expression in the eye through the regulation of the eye specific promoters GMR and eyeless, we can study the genetic interactions of Baboon (TGF- β/Activin like TGF-βII receptor) with components of PI3K signaling. I will examine this genetic interaction using scanning electron microscopy (SEM). These experiments will determine if there is an interaction with dFOXO, dPI3K, dAkt and Shaggy (GSK-3β) with the Baboon receptor.

Chapter 2 Materials and Methods

2.1 Cell Culture

All cell cultures was performed at 37^0C in a 5% CO_2 humidified atmosphere. Two phenotypically normal fibroblast cell culture lines (AKR-2B and NRK-49) were grown in high-glucose Dulbecco's modified Eagle medium (DMEM, Invitrogen, Canada) supplemented with 10% fetal bovine serum (FBS). NIH-3T3 fibroblasts grown in high glucose DMEM were supplemented with 10% Normal Bovine Calf Serum (NBCS). Two phenotypically normal epithelial lines (mink lung epithelium, Mv1Lu and a mouse mammary gland epithelium, NMuMG) were grown in DMEM/10% FBS, with the media of the NMuMG line supplemented with 10 μg/ml bovine insulin (Sigma) and 5 ng/ml recombinant human EGF to promote growth and prevent differentiation. Chimeric receptor expressing clones have been described previously (Anders and Leof, 1996; Dore *et al.*, 2001). Briefly, the ligand-binding domain of GM-CSF was fused to the TGF-β transmembrane and cytoplasmic domain to create receptors that respond to GM-CSF with a TGF-β signaling cascade. I utilized three lines, as outlined in table 1: 1) Contains both normal Type I and Type II TGF–β receptors , 2) Type I kinase dead (K232R) with a normal Type II TGF-β receptor or 3) Type II kinase dead (K227R) with a normal Type I TGF-β receptor. This allows for use of the endogenous TGF-β receptors as a control, as the chimeric receptors do not interact with the normal TGF-β receptors. Chimeric AKR-2B fibroblast lines were maintained in 10% FBS in McCoy's 5A medium (Invitrogen,

Table 2: Characteristics of TGF-β chimeric receptor lines

Clone	Cell Type	Inactivating Mutation
AHM-4	Fibroblast	Express Normal Chimeric receptors
A708	Fibroblast	Type I Kinase Dead (K232R)
A618	Fibroblast	Type II Kinase Dead (K227R)

Canada) and supplemented with 100 μg/ml geneticin and 50 μg/ml hygromycin B (Sigma Canada) to maintain the chimeric receptors expression. Fibroblast cell lines were plated at $1.5x10^5$ cells per well in each well of a six well dish in growth media and allowed to attach for 24 hours. They were serum deprived overnight in 0.1% NBCS/DMEM prior to treatment with TGF-β2 (1-5 ng/ml) for indicated time periods (0-6 hours). For epithelial cells, Mv1Lu were plated at $3.0x10^5$ cells per well, allowed to attach for 24 hours and treated as indicated. NMuMG cells were plated at $1.0x10^5$ per well in six well plates, allowed to attach for 24 hours, growth factor deprived for ~16 hours in DMEM/10% FBS and treated as indicated. Inhibitors used in this study were dissolved in DMSO and stock solutions were stored at -20°C and protected from light. LY290004 (Cell Signalling, Invitrogen, Canada) was used at a final concentration of 10 μM (10mM stock), and Akt inhibitor IV (Calbiochem, USA) was used at a concentration of 1 μM (stock 1mM).

2.2 Protein Extraction

After cells were treated for the indicated time periods, plates were placed on ice and wells were washed 3x in ice-cold PBS. Total cellular protein was obtained by lysing cells in a modified ice-cold RIPA buffer (PBS, 50 mM Tris-HCl pH 7.4, 0.5% Sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 50 mM β-Glycerol -phosphate, 5 mM EDTA, 100 μM Na_3VO_4, 50 μg/ml of phenylmethylsulfonyl fluoride and 1x Complete protease inhibitor cocktail [Roche Canada]) for 5 minutes on ice. Cellular lysates were then collected using cell lifters (Fisher Canada) and transferred into 1.5ml microcentifuge tubes. Cellular debris were pelleted by centrifugation at 21,000x g for 10 minutes at 4^0C and supernatant was transferred to a new 1.5ml microcentifuge tube. Cell lysates were then stored at -80^0C.

Nuclear and cytoplasmic extracts were prepared (N-PER, Pierce) as described by the manufacturer's protocol from AKR-2B and NMuMG cells treated with 2ng/ml TGF-β2 to assay Cyclin D1 distribution using Western blotting. Total protein in all samples was determined using the BCA protein assay kit (Pierce) according to the manufacturer's protocol and read at 562 nm on a Polar Star plate reader (BMG Inc) using bovine serum albumin as a standard.

2.3 Immunoprecipitation

Protein was extracted from NMuMG and AKR-2B as described previously in a RIPA buffer modified for immunoprecipitation (1x PBS, 50 mM Tris-HCl pH 7.4, 1% Triton X-100, 50 mM NaF, 50 mM Glycerophosphate, 10 mM EDTA, 1 mM Na3VO4, 50 μg/ml of phenylmethylsulfonyl fluoride and 1x Complete protease inhibitor cocktail). Equivalent amounts of total protein were diluted with fresh IP buffer to an equal final volume, and monoclonal anti-Cyclin D1 (Cell Signalling) was used to immunoprecipitate the protein overnight (~16 hours) at 4^0C on a rocker platform. Protein G-sepharose (Pierce) was added for 2 hours with rocking to collect the antibody/Cyclin D1 complexes. Protein G/Antibody complexes were centrifuged at 8000x g for 1 minute to pellet the Sepharose beads. Supernatant was aspirated using a 27g needle to prevent bead loss, and the Protein G/Antibody complex was washed ice-cold IP buffer rocking at 4^0C for 10 minutes. This was repeated 3x, followed by one wash with PBS. Samples were then resuspended in 2x Electrophoresis sample buffer, vortexed, and heated for 3 minutes at 100^0C. Samples were separated by SDS-PAGE, and then transferred to PDVF membrane and co-precipitated CDK-4 detected using an anti-CDK-4 antibody (Cell Signaling) to determine the amount of CDK4/Cyclin D1 complex formation.

2.3 Western Blotting

Electrophoresis sample buffer (5x strength, 5 % SDS, 150 mM Tris HCL pH 6.8, 5% glycerol, 5% β-mecaptoethanol and 0.125 % Bromophenol blue) was added to each protein extract and boiled for 3 minutes. Equivalent mass of total protein sample was then resolved on the appropriate 8.5 or 12.5% acrylamide gel in either a Protean II minigel apparatus (BioRad) or an ADT-26 gel apparatus (Owl Scientific). Gels were transferred for 1 hour at 100 volts using a Transblot apparatus (BioRad) to either PDVF or Nitrocellulose membranes and specific proteins were detected with antibodies as indicated. Immune complexes detected using HRP-conjugated species-specific antibodies (Santa Cruz, HRP-anti-mouse or HRP anti-rabbit) followed by ECL detection (Pico Supersignal, Pierce). Specific protein from each sample was probed with a phospho-specific antibody (Akt Serine 473, PDK-1 Serine 241, GSK-3β Serine 9, Cyclin D1 Theronine 286, Cell Signalling), stripped for 30 minutes at 55^0C in buffer (100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH6.5) , and tested with the corresponding antibody for total specific protein (PDK-1, Akt, Cyclin D1 (DC6) Cell Signaling or GSK-3β, Cyclin D1, Santa Cruz). Other antibodies used in this studied were CDK-4 (Cell signaling).

2.5 Morphological transformation

AKR-2B cells were plated at $2.5x10^5$ cells per well of a six well dish, allowed to grow to confluence and then treated with serum-free Iscove's medium (Invitrogen, Canada). After 48 hours, either medium alone was added or medium containing inhibitors (Akt inhibitor IV, Calbiochem) with or without 5ng/ml TGF-β2 as indicated for an additional 48 hours, with fresh inhibitor and TGF-β being added at 24 hours. After

treatment, cells were washed 2x with room temperature PBS and fixed for 30 minutes at 4 degrees C in 4 % Paraformaldehyde (PFA) in PBS. After fixation, cells were washed once in PBS, permeabilized in 0.5% Trition in PBS for 90 seconds, then washed twice for 5 minutes with PBS. FITC-conjugated phalloidin (Sigma, Canada) was then added for 40 minutes at a dilution of 1/500 and cells were washed extensively in PBS. Images were captured using a Leica DMRIEZ inverted microscope using a 20x Pau Lyedtide brightfield phase contrast optics while fluorescence images of phalloidin staining used an L5 filter set.

2.6 Growth Assays

Growth assays were undertaken as previously described (Wilkes *et al.*, 2003) with minor modifications. Briefly, NIH-3T3 cells were plated at 50,000 cells per well, allowed to attach 24 hours, serum deprived in 1% NBCS/DMEM for 24 hours. This medium was replaced with medium containing 5 ng/ml TGF-β with or without Akt IV inhibitor. After incubation for 18 hours, H^3-thymidine (1 μCi) was added for 2 hours. The assay was stopped by washing cells 2x for 10 minutes with 4°C 10% TCA and H^3-thymidine incorporation determined by solubilizing the precipitated DNA with 0.2M NaOH, 0.2 mg/ml sheared salmon sperm DNA and counting an aliquot (100μl) in a scintillation counter (Beckman).

2.7 Drosophila Culture

Flies were cultured on standard cornmeal/molasses/yeast media. All experiments were done at 25°C. We utilized the UAS/Gal4 system of ectopic gene expression (Phelps and Brand, 1993) for misexpression in the developing eye, and Table 2 illustrates the corresponding fly and mammalian names. *Glass multiple reporter (GMR-Gal4)/cyo*

females were crossed to *UAS-dActivin-β$^{dsRNA.HL}$ / UAS-dActivin-β$^{dsRNA.HL}$* and *UAS-babo/UAS-babo* and *w1118* (Control) males. *Eyeless- Gal4/cyo, Eyeless-Gal4; UAS-HA-PKB (HA Akt)/cyo* and *Eyeless- Gal4; UAS-GSK-3β/cyo* females were crossed to *UAS-babo/UAS-babo, UAS-Babo^{QD} (UAS BaboQ302D)/ UAS-Babo^{QD}* and *w1118* (Control) males. *Eyeless-Gal4; UAS-PI3KDN (UAS-Dp110^{D954A})/cyo* females were crossed to *UAS-Babo^{QD}* and *w1118* (control) males. *GMR-GAL4/cyo; UAS- Babo^{QD}*/TM3 flies were generated from the original line (UAS Babo^{QD}) using standard genetic means. *UAS-PI3K (UAS-Dp110), UAS-PI3K^{DN} (UAS-dp110^{D954A}), UAS-Dakt, UAS-dFOXO, UAS-mFOXO (murine FOXO), UAS-mFOXO^{AA} (mFoxo1$^{T24A/S253A}$) , UAS-sgg (UAS-sgg^{5xHisTag}), UAS sgg^{S9A} and UAS-sgg^{KD}* (UAS-sgg^{K83M}) males were crossed to *GMR-GAL4; w1118* (control) and *GMR GAL4; UAS- BaboQD* males.

Driver lines *Eyeless- Gal4* and *Glass multiple reporter (GMR-Gal4)* were obtained from the Bloomington stock center, along with *UAS-sgg, UAS sgg^{S9A} and UAS-sgg^{KD}. UAS-Dakt* is described in Staveley, *et al.*, (1998). *UAS-dFOXO, UAS-mFOXO* and *UAS-mFOXO^{AA}* are described in Kramer *et al.*, 2003. *Eyeless-Gal4; UAS-HA-PKB (HA Akt)/cyo* and *Eyeless- Gal4; UAS-GSK-3β/cyo* were generated by Staveley, unpublished. *UAS-PI3K^{DN}* and *UAS-PI3K* were generously provided by Dr. Sally Leevers (1996). *UAS-dActivin-β$^{dsRNA.HL}$* , *UAS-babo^{QD}* and *UAS- babo* were generously provided by Dr. Mike O'Connor (Brummel *et al.*, 1999).

Table 3: Drosophila and Mammalian Names

Drosophila Homologue	Mammalian Homologues
dActivin	Activin
Baboon (Babo)	Type I Activin/TGF-β Receptors (ALK)
Dp110, dPI3K	PI3K
dAkt	Akt 1, 2, 3
dFOXO	FOXO1, 3, 4
Shaggy	GSK-3α/β

Analysis of Adult Eyes

Flies were selected via markers for the desired genotypes, and frozen at –70°C in a 95 % ethanol bath to await further analysis. For scanning electron microscopy (SEM), two-day-old females were coated in gold and desiccated overnight as described by Kramer and Staveley (2003). Pictures were taken at 150x magnification using a SEM and a Hitachi camera. Negatives of the SEM pictures were scanned into Adobe Photoshop, and contrast/intensity/brightness was adjusted for each picture individually to obtain a clearer image. All pictures were cropped such that there was no change in relative magnification to facilitate comparison of eye size. All analyses were done on a minimum of three separate animals.

Statistics

All experiments were repeated in triplicate. For Drosophila experiments, a minimum of 6 flies was examined by SEM, and three images were captured. Calculations for the growth assays were done using Microsoft Excel spreadsheets and graphs were generated using GraphPad Prism 4. One way ANOVA analyses for growth assays were performed using GraphPad Prism.

Chapter 3: Results

Chapter 3.1 Fibroblast Signalling

To investigate the molecular signalling pathways of TGF-β, I wanted to describe Smad-independent pathways involved in cell cycle and growth regulation of fibroblasts. Given prior evidence of TGF-β signalling through Akt (Shin *et al.*, 2001) and this protein's well-characterized involvement in growth and cell cycle, an interaction with Akt pathway components was examined in fibroblast cells. I first wished to look at the activation of PDK-1, a kinase responsible for the phosphorylation of T-308, a phosphorylation site necessary for the activation of Akt (Figure 3A). A time course in AKR-2B cells demonstrates that there is an increase in PDK-1 Serine 241 phosphorylation by 60 minutes that continued through 180 minutes. Next, we wanted to examine the temporal effects of TGF-β on Akt activation. We were unable to examine phosphorylation at the T-308 site, as the antibody was unfeasible in both TGF-β and EGF positive control treated cells (data not shown). Akt protein was analyzed for changes in phosphorylation (P-Akt, Serine 473) by Western blotting. Varying times of stimulation by TGF-β determined optimal timing for later experiments. In fibroblast cells (Figure 3B), there is an increase in S-473 phosphorylation starting at 60 minutes and increasing through 180 minutes. This was replicated in triplicate in 3 fibroblast cell lines to ensure that the phenomenon examined was not specific to a particular cell line. As well, we wished to evaluate the interaction of PI3K and the TGF-β receptors in Akt phosphorylation in fibroblasts. The PI3K specific inhibitor, LY29004, was used at a 10μM concentration to minimize non-specific effects such as non-specific inhibition of

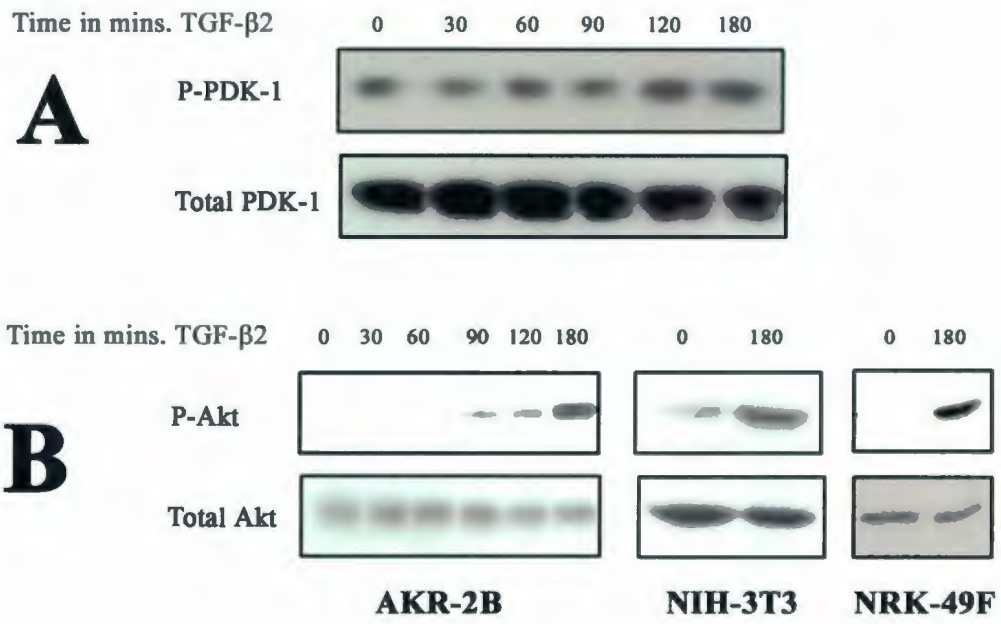

Figure 3: TGF-β induced PDK-1 and Akt phosphorylation is increased in fibroblasts

Fibroblast cells were sparsely plated and serum-depleted for 16 hours prior to stimulation with 1ng/ml TGF-β2 for indicated time points. (A) PDK-1 phosphorylation is increased by TGF-β treatment in AKR-2B cells. Temporal effects of TGF-β on PDK-1 were assayed for changes in phosphorylation at Serine 241 in AKR-2B cells. Total PDK-1 was then probed as a loading control. (B) TGF-β activates Akt in fibroblast cells. Temporal effects of TGF-β on Akt activation were analyzed for changes in phosphorylation (P-Akt, S-473) by Western blotting. In fibroblast cells (AKR-2, NIH-3T3 and NRK-49F), an increase was noted by 180 minutes. Total Akt was probed as a loading control and all experiments were repeated in triplicate.

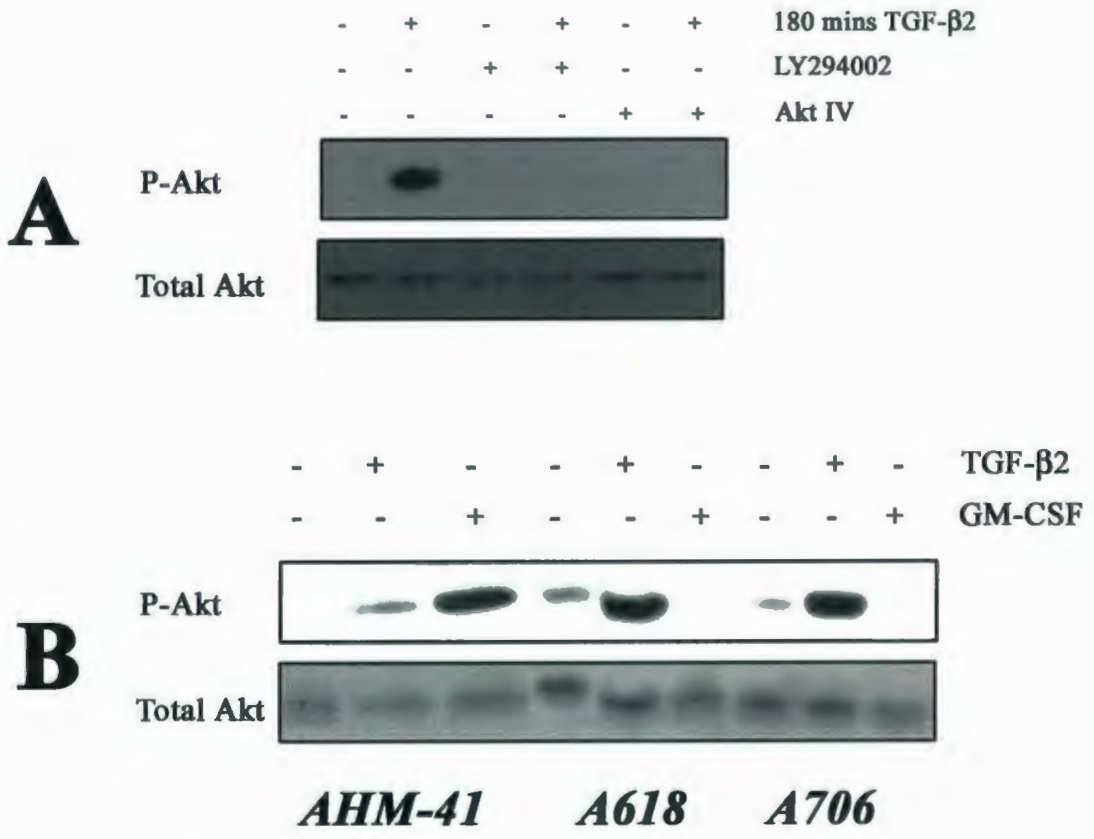

Figure 4: TGFβ induced Akt phosphorylation is dependent on both receptors and PI3K.

Cells were plated at low density and serum-depleted for 16 hours prior to stimulation. (A) Akt phosphorylation is dependent on PI3K activity. AKR-2B cells were stimulated with or without 2ng/ml TGF-β2 for 180 minutes in the presence/absence of 10μM LY29004 or 1 μM Akt IV inhibitors. Phosphorylated Akt (S-473) was then assayed by Western Blotting, with total Akt probed as a loading control. (B) Akt phosphorylation requires activity of both TGF-β receptors. Stable chimeric receptor lines of wild type (AHM-4), the TGF-β Type I (A618) and II (A706) kinase dead receptors were stimulated for 120 minutes with 5ng/ml TGF-β2 or GM-CSF as indicated. Akt phosphorylation was then assayed by Western Blotting, with total Akt being probed as a loading control. All experiments were repeated in triplicate.

inhibitors reduced S-473 phosphorylation of Akt in response to TGF-β stimulation (Figure 4A), indicating that TGF-β actions on Akt is through activation of PI3K.

The ability of the TGF-β ligand to signal requires two receptors, the TGFβRI and TGFβRII . To study the role of the receptors in the activation of Akt, we used a chimeric TGFβ receptor model system as described by Anders and Leof (Anders *et al.*, 1996). This allows for the creation of receptors that only respond to GM-CSF, while leaving the native TGFβ receptor system intact and responsive to TGFβ as an excellent internal control. To show if the kinase activity of the receptors was essential for Akt phosphorylation we utilized 3 stable clonal lines: one that expresses both wild type receptors (AHM-4), one that expresses a kinase dead Type I (A706) and one that expresses a kinase dead Type II (A618). As expected, the signal generated by GM-CSF, relative to TGFβ, was greatly increased, likely due to overexpression of chimeric receptors (Figure 4B). Both of the kinase dead lines did not display an increase in phospho-Akt in response to GM-CSF, indicating that the kinase activity of both type I and II TGFβ receptors is essential for Serine 473 Akt phosphorylation. The normal endogenous response to TGFβ was not impaired in these cell lines, indicating there is not impairment in the ability of TGFβ receptors to activate Akt in these cell lines.

Since we were especially interested in the growth promoting and cell cycle effects of TGF-β, we picked GSK-3β for further analysis. GSK-3β has been previously shown to be involved in both growth and cell cycle (Doble *et al.*, 2003). In fibroblast cells (AKR-2B, NRK-49F and NIH-3T3), there is an increase in serine 9 phosphorylation by 90 minutes increasing through 180 minutes (Figure 5A, B). These results correlate well with

the timing of Akt activation, as Akt is responsible for Serine 9 phosphorylation of GSK-3β, inactivating the protein kinase activity. This action should potentially lead to a decrease in phosphorylation of GSK-3β targets. This Serine 9 phosphorylation event of GSK-3β was also dependent on both PI3K and Akt activity as both LY294002 and Akt inhibitor IV, blocked TGF-β mediated increases in GSK-3β phosphorylation (Figure 5C). This indicates that GSK-3β phosphorylation by TGF-β stimulation is dependent on both PI3K and Akt activity.

It has been previously shown that GSK-3β can phosphorylate Cyclin D1 at Thr 286 (Diehl et. al., 1998) resulting in cytoplasmic relocalization, disruption of binding to CDKs and degradation. TGF-β can regulate G1/S progression and regulate Cyclins via an unknown mechanism (Laiho *et al.*, 1990). We hypothesize that in response to TGFβ stimulation, GSK-3β is inactivated in fibroblasts altering Cyclin D1 activity. Cyclin D1 phosphorylation was examined by Western blotting and found to decrease by 180 minutes following TGF-β addition (Figure 6A). This time point coincides with increased in P-GSK-3β and therefore inactive GSK-3β. Additionally, nuclear extracts from TGF-β treated cells revealed Cyclin D1 localizes to the nucleus, consistent with the known activity of unphosphorylated Cyclin D1 (Figure 6B). In addition to the cellular location of cyclin D1, binding to CDK-4 is necessary for proper functionality. As shown in Figure 6C, stimulation with TGF-β alone cause increased complex formation, and LY20004 blocked this event. This suggests that TGF-β is able to directly regulate the Cyclin D1/CDK complex formation in a PI3K dependent manner.

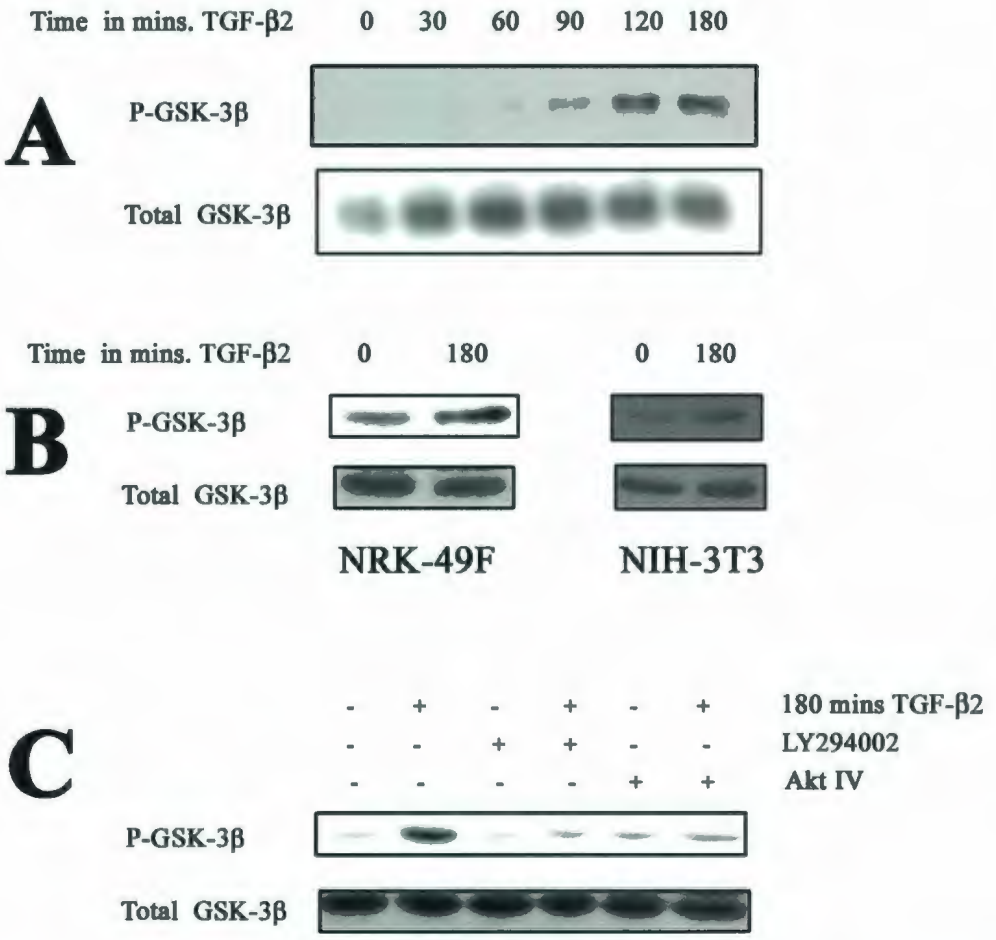

Figure 5: GSK-3β phosphorylation is increased and dependent on PI3K and Akt.

Fibroblast cells were plated at low density, serum depleted for 16 hours, and stimulated with TGF-β2 for indicated time points. TGF-β inactivates GSK-3β in Fibroblast cells. (A) Temporal effects of TGF-β on GSK-3β inactivation were analyzed for changes in phosphorylation after 1 ng/ml TGF-β2 stimulation (P-GSK-3β, S-9) by Western blotting. Total GSK-3β was probed as a loading control (B) In other fibroblast cells (NIH-3T3 and NRK-49F), there was an increase by 180 minutes. (C) Phosphorylation of GSK-3β is dependent on both PI3K and Akt. AKR-2B cells were stimulated with or without 2ng/ml TGF-β2 for 180 minutes in the presence/absence of 10μM LY29004 and 1 μM Akt IV inhibitors. Phosphorylated GSK-3β (S9) was then assayed by Western Blotting, with total GSK-3β probed as a loading control. All experiments were done in triplicate.

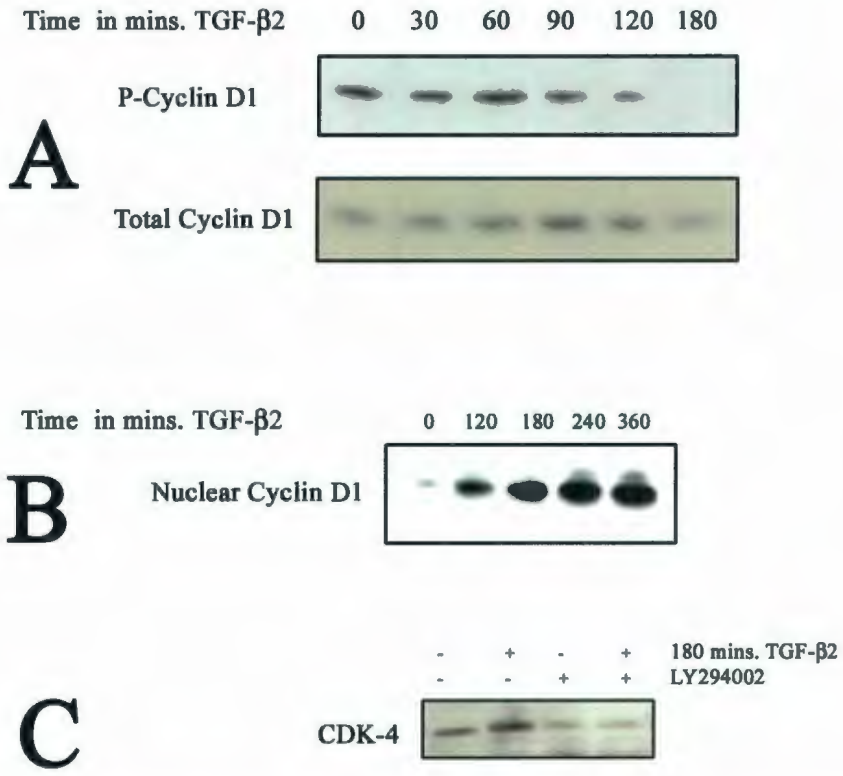

Figure 6: TGF-β induces decreased phosphorylation and nuclear localization of Cyclin D1.

Fibroblast cells were seeded at low density, serum depleted for 16 hours, and stimulated with TGF-β2 for indicated time points. (A) Temporal effects of TGF-β on Cyclin D1 phosphorylation were analyzed for changes by Western blotting, with total Cyclin D1 being probed as a loading control. There is a decrease in Cyclin D1 phosphorylation at the GSK phosphorylation site (T-286) by 90 minutes. (B) Nuclear extracts were prepared and assayed for Cyclin D1 showing localization to the nucleus with TGF-β treatment. (C) Correlating with this localization event, increased complex formation of Cyclin D1 with CDK-4 was observed and was shown to be PI3K dependent.

Having possibly defined a TGF-β/Akt pathway signaling mechanisms of fibroblasts, we wished to determine the biological effects controlled by this novel pathway. To look at the role of Akt activation in regulation cytoskeleton and cellular morphology, we undertook a morphological transformation assay. Fibroblast cells that are stimulated with TGF-β will take on an elongated, myofibroblast appearance with actin stress fibers forming along the long axis of cells when compared to untreated controls (Figure 7, panel B and A respectively). In addition to the standard brightfield photomicrographs, cells were stained with FITC labelled phalloidin, which detects actin filaments (filamentous actin). When Akt IV inhibitor is added to the media with TGF-β, the elongated cell morphology was prevented (Figure 7 panel C and D), however there is still evidence of actin stress fiber formation.

As well as causing morphological transformation, TGF-β can induce growth in fibroblasts. To study the role Akt plays in TGF-β mediated growth, we utilized a ^{3}H-thymidine assay to measure DNA synthesis. This is an indirect measure of growth, as it is assumed that cells undergoing DNA synthesis progress to mitosis to give increased cell number. We found that inhibition of Akt blocked TGF-β mediated growth (Figure 8). A significant difference was found between TGF-β and Akt/TGF-β treated conditions ($p<0.001$, One way ANOVA, corrected for multiple comparisons using Bonferroni t-test), with no difference being found between the untreated controls and inhibitor alone.

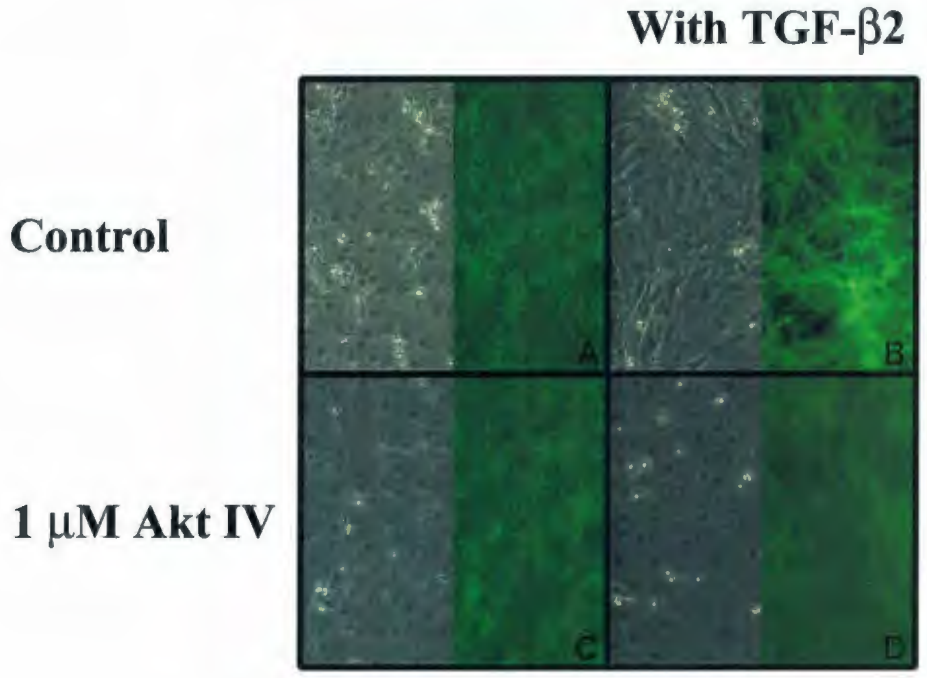

Figure 7: Inhibition of Akt attenuates TGF-β induced morphological transformation.

AKR-2B cells were grown to confluence then placed in serum free media for 48h and Akt inhibitor was added to cells with or without 5 ng/ml TGF-β. The cells treated with inhibitor alone were similar to control untreated cells. In the upper right panel (B), the morphological transformation event can be seen as elongated cells when compared to the control (A). When both the inhibitor and TGF-β are present, there is little transformed phenotype (D). This suggests that Akt plays a role in TGF-β induced morphological transformation.

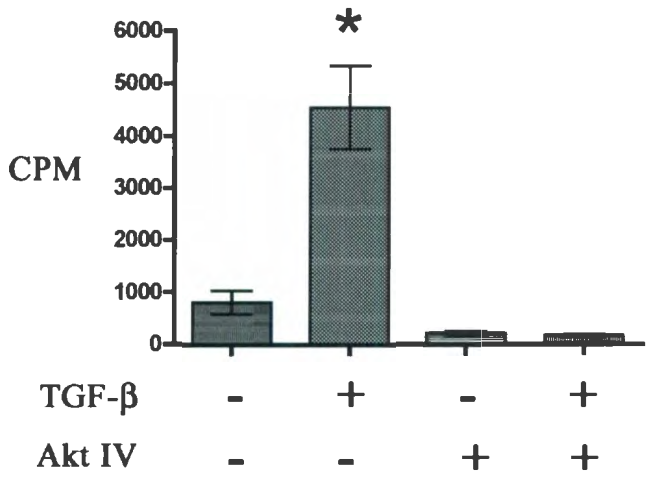

Figure 8: Inhibition of Akt reduces TGF-β induced growth.

NIH-3T3 cells were plated, allowed to attach 24 hours, and then stimulated with 5 ng/ml TGF-β2 with or without Akt IV inhibitor and ^{3}H-thymidine incorporation measured. The cells treated with inhibitor alone were similar to control untreated cells. When both the inhibitor and TGF-β are present, there was no increase in growth, as with TGF-β alone. This suggests that Akt is involved in TGF-β induced mitogenesis of fibroblasts. A star indicates the statistically significant treatment.

Chapter 3.2 Epithelial Signalling

Since fibroblasts are growth stimulated by TGF-β and this response requires Akt, we wished to examine TGF-β signalling in epithelial cells. TGF-β has known differential effects on growth/cell cycle between fibroblasts and epithelial cells, causing a G1/S block and decreased growth in epithelial cells and increased G1/S progression and growth in fibroblast (Laiho *et al.*, 1990). Since the Smad signalling pathway is the same in both cell types (Massague, 2000), and Pak2 is active only in fibroblasts we wished to extend these findings to Akt signalling (Wilkes et al., 2003).

We examined PDK-1 phosphorylation (Figure 9A) and found that this event is decreased by 90 minutes in the NMuMG cell line. When Akt phosphorylation is examined in response to TGF-β (Figure 9 B, C) in epithelial cells, there is a decrease by 90 minutes, with a loss of phosphorylation seen by 120 minutes. Again, we examined two cell lines (Mv1Lu and NMuMG) to ensure this is not a cell line specific event. In the epithelial lines, a slight difference is noted that the NMuMG line seems to respond 'faster' than the Mv1Lu line (Figure 9 B, C). However, as the Mv1Lu are grown in their complete media, this difference in signaling could be due to other growth factors increasing the background of Akt phosphorylation.

We also wished to establish if there was any potential differences in GSK-3β phosphorylation with TGF-β stimulation. Figure 10 shows that phosphorylation of GSK-3β at S-9 is decreased on a similar time frame to Akt. Again this was replicated in two different epithelial cell lines to ensure this is not a cell line specific event. This suggests that as Akt becomes dephosphorylated (and therefore inactive) it is unable to

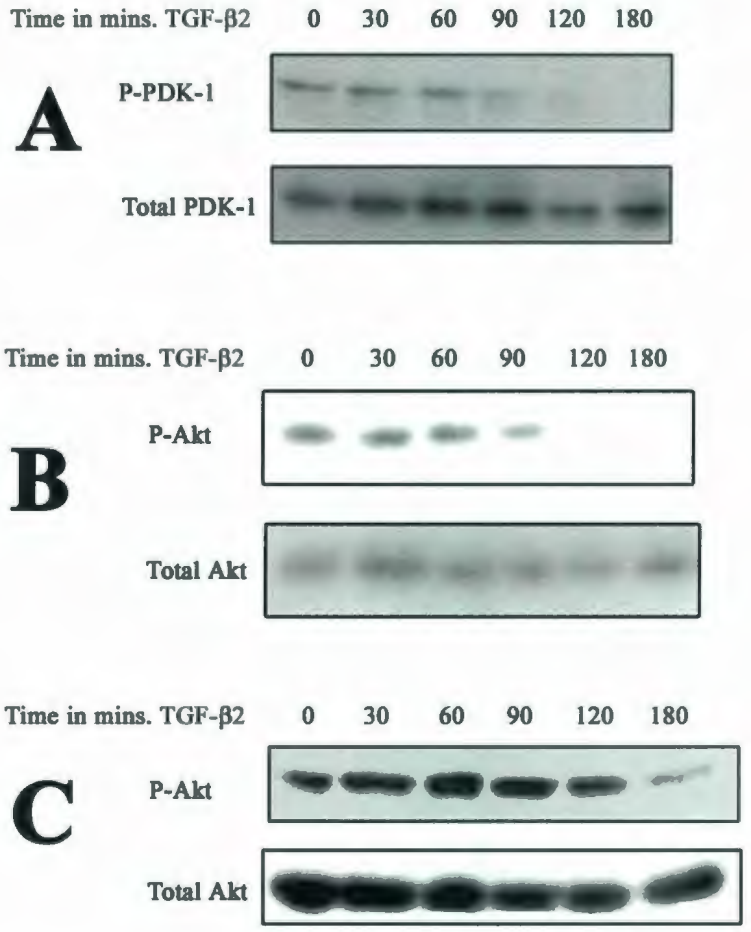

Figure 9: TGF-β decreases Akt and PDK-1 phosphorylation in epithelial cells

Epithelial cells were plated, allowed to attach for 24 hours, growth factor depleted for 16 hours and stimulated for the indicated time points with 1 ng/ml TGF-β2.
(A) PDK-1 phosphorylation is decreased by TGF-β treatment in NuMG cells. Temporal effects of TGF-β on PDK-1 (Serine 241) phosphorylation were assayed for changes in phosphorylation in NuMG cells, with total PDK-1 being blotted as a loading control. There was a decrease in phosphorylation at 180 minutes. (B) TGF-β decreases Akt phosphorylation in NuMG cells. Phosphorylation of Akt (S-473) was assayed for changes, with total Akt being blotted for a loading control. TGF-β caused a decrease by 120 minutes, with little phosphorylation left at 180 minutes. (C) Mv1Lu cells also display decreases phosphorylation of Akt with TGF-β stimulation over a similar time frame.

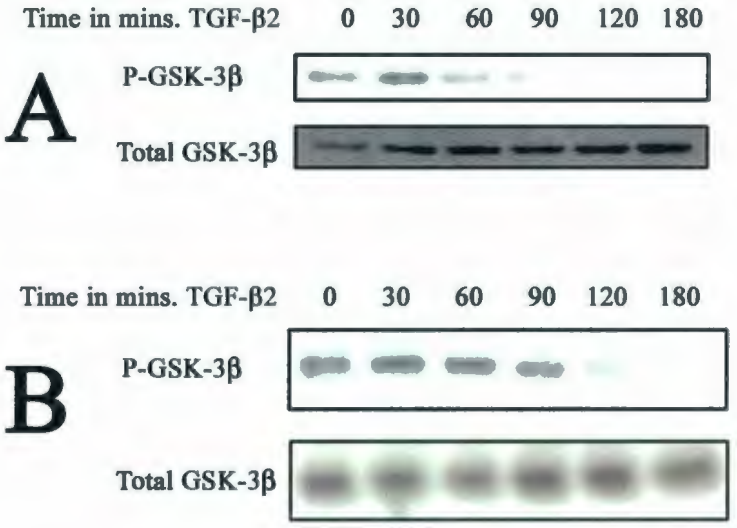

Figure 10: GSK-3β phosphorylation is decreased in epithelial cells in response to TGF-β stimulation.

(A) TGF-β decreases phosphorylation of GSK-3β in NuMG cells. Epithelial cells were seeded, allowed to attach for 24 hours, growth factor depleted in 10% FCS/DMEM for 16 hours and stimulated for the indicated time points with 1 ng/ml TGF-β2. Between 60 and 90 minutes there is a decrease in phosphorylation of GSK-3β (Serine 9), with no detectable phosphorylation by 180 minutes. (B) MvLu cells also display decreases phosphorylation of GSK-3β with TGF-β stimulation over a similar time frame.

phosphorylate GSK-3β at Serine 9.

Since TGF-β causes a G1/S block in epithelial cells, we wished to examine the effects on Cyclin D1 since this pathway could explain how TGF-β mediates this action. Surprisingly, we found no increase in phosphorylation of Cyclin D1 as would be expected if more active GSK-3β is present in the cell (Figure 11A). However, we noted a decrease in the total Cyclin D1. Since phosphorylation of Cyclin D1 causes rapid degradation of the protein, perhaps this is why we did not detect an increase. We also examined the effects of TGF-β on Cyclin D1 localization, and found that the total protein levels were decreased in both cytoplasmic and nuclear fractions (Figure 11B). This is consistent with the idea that TGF-β is causing the breakdown of Cyclin D1. When we examined complex formation, we found that TGF-β decreases the amount of CDK-4 that is found in complex with Cyclin D1 (Figure 11C). All together, this data suggests that TGF-β can: 1) decrease the amount of Cyclin D1 in the cell and 2) inhibit the function of Cyclin D1/CDK-4 complexes. Both of these events have been shown to slow the progression through G1/S.

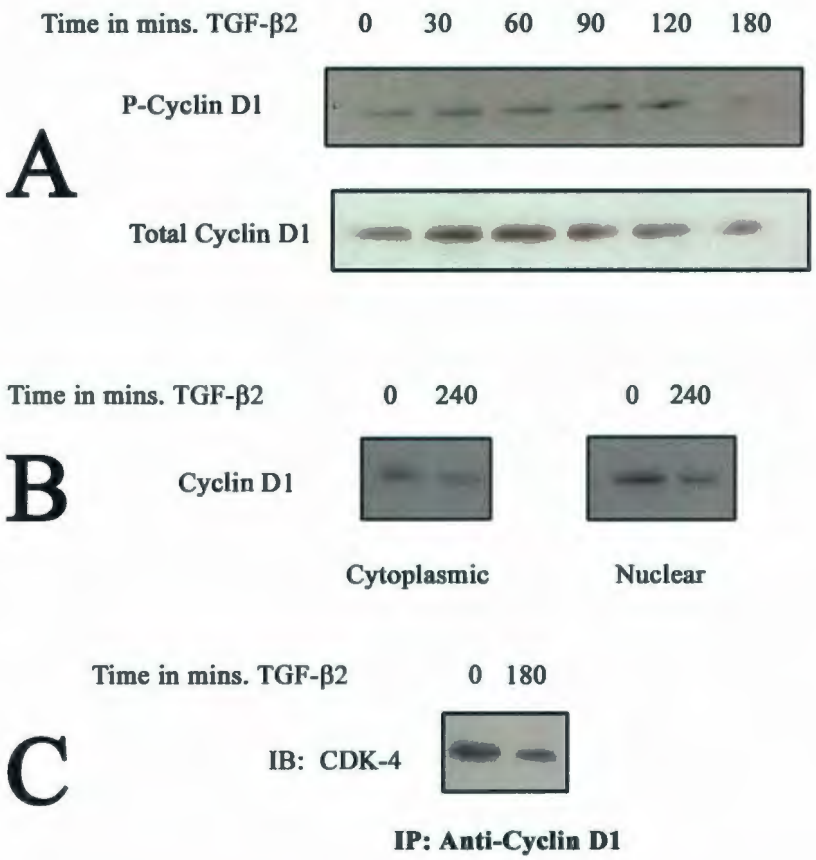

Figure 11: TGF-β decreases protein levels of Cyclin D1 and association with CDK-4.

Epithelial cells (NuMG) were plated, allowed to attach for 24 hours, growth factor depleted for 16 hours and stimulated for the indicated time points with 1 or 2ng/ml TGF-β2. (A) Temporal effects of TGF-β on Cyclin D1 phosphorylation were analyzed by Western blotting. There is no change in Cyclin D1 phosphorylation at the GSK phosphorylation site (T-286) by 120 minutes, with total protein levels being corresponding decreased (B) Nuclear extracts were prepared and assayed for Cyclin D1 showing decreases in protein levels in both the cytoplasmic and nuclear fractions with TGF-β treatment. (C) Correlating with this loss of total Cyclin D1, decreased complex formation of Cyclin D1 with CDK-4 was observed.

Chapter 3.3 Drosophila Model of TGF-β/Akt signaling

In addition to our *in vitro* signaling model in cell culture, we wished to determine if there is a genetic interaction between TGF-β signalling and the Akt pathway in Drosophila. To accomplish this aim, we used the UAS/Gal4 ectopic system of gene expression (Brand and Permmion, 2003) to overexpress components of TGF-β and Akt signalling in the developing eye. This system was developed from yeast, utilizing the Gal4 transcription factor that binds to a specific upstream activator sequence (UAS). One line is the 'driver' line that contains a promoter upstream of GAL4. The second line contains the UAS upstream of a gene product. When the lines are crossed together, the Gal4 binds to the UAS sequence to express the gene product in a temporal and spatial manner. This system has the advantage that lethal mutations can be expressed in a tissue specific manner, thereby bypassing early developmental roles of the protein that may cause lethality. Also, UAS sequences are not present in the Drosophila genome, ensuring there is no non-specific expression of the gene.

In our study we utilized two driver lines: *eyeless-Gal4* and *GMR-Gal4*. *Eyeless-Gal4* is expressed early in the eye primordia, before photoreceptor differentiation and continues expression until the completion of eye development. It is commonly used to determine if a gene product is necessary for patterning decisions in the eye. *GMR-Gal4* specifically activates gene expression within and posterior to the morphogenetic furrow during late eye development allowing the study of cellular growth decisions distinct from early eye patterning. The driver lines were also crossed to w^{1118} as a control. As high levels of the GAL4 protein have been shown to cause apoptosis and developmental

defects in the developing eye (Kramer and Staveley, 2003), this control is necessary for comparison to ensure no off-target effects.

For our first set of experiments, we wished to examine the TGF-β Activin/TGF-βRI homologue in flies, Baboon and its ligand dActivin. Expression of the normal Baboon receptor under both the control of the *eyeless* and *gmr* promoter has little effect on growth of the eye as determined by visual comparison (Figure 12 B and E respectively, compared to 12 A and D), with perhaps slightly increased bristle size being the only phenotype seen. Since the normal Baboon receptor is highly overexpressed compared to levels of both the endogenous ligand (dActivin) and its type II receptor (Punt), it is not surprising that little effect was seen with this construct. Also, Brummel *et al.*, 1999 did not see any effect on wing size when overexpressing this construct. In addition, we misexpressed a dominant negative construct of dActivin and saw little effect on the size or patterning of the eye (Figure 12B compared to the control 12A). When the Baboon receptor was expressed in conjunction with Akt, a small increase in the total eye size may be present (Figure 12 F and G). Expression of the wt Baboon receptor in conjunction with human GSK-3β had no effect (Figure 12 H and I). In all, overexpression of the wildtype receptor or inhibiting the wildtype ligand had no discernable effect on eye development.

We also misexpressed an activated form of the Baboon receptor ($Baboon^{QD}$). This mutation is in the glycine-serine rich (GS) domain of the receptor. The GS domain is where the Type II TGF-β receptors phosphorylated the Type I receptors to activate kinase activity, and therefore downstream signalling. $Baboon^{QD}$ contains an amino acid change at position 302 from glutamine to aspartic acid, to produce a constitutively active

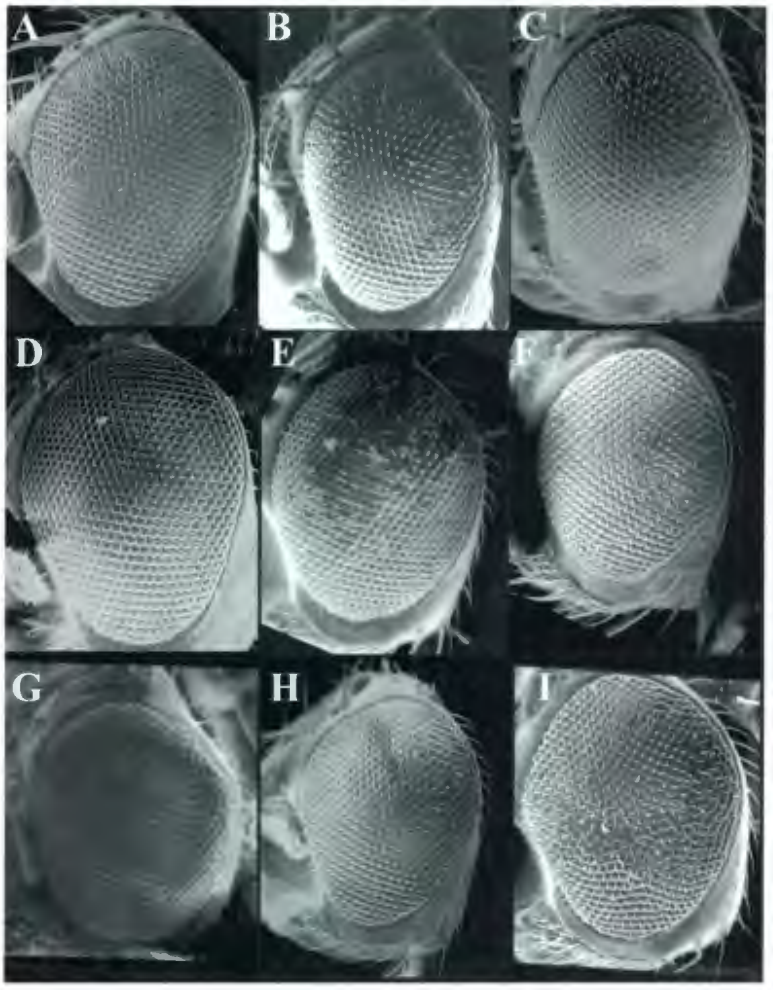

Figure 12: Overexpression of normal TGF-β components reveals no phenotype.
Overexpression of either dActivin (B) or Baboon (C) had little effect on eye development with GMR-Gal4 compared to GMR-Gal4 flies alone (A). Expression of Baboon with eyeless driver (E) resulted in slightly longer bristles when compared to wildtype (D). When coexpressed with GSK (G) had little effect when compared to GSK (F) alone. When coexpressed with Akt (I) a slightly larger eye is noted, when compared to Akt alone (H) or eyeless Gal4 alone (D).
(A) *GMR-Gal4/+* (B) *GMR-Gal4/+; UAS-dActivin/+* (C) *GMR-Gal4/+; UAS-babo/+* (D) *eyeless-Gal4/+* (E) *eyeless-Gal4/+; UAS-Babo/+* (F) *eyeless-Gal4; UAS-Akt* (G) *UAS-babo/eyeless-Gal4; UAS-Akt* (H) *eyeless-Gal4; UAS-wtGSK-3β/+* (I) *UAS-babo/eyeless-Gal4; UAS-wtGSK-3β*

receptor, presumably through an increase in kinase activity of the receptor (Brummel *et al.*, 1999). When we expressed this form of the receptor with the *eyeless* promoter, we saw severe outgrowth from the eye (Figure 13 A, E). When coexpressed with wildtype Akt, we saw a synergic effect, which would be expected if the normal function of this receptor were to activate Akt (Figure13 B, F). Loss of dPI3K in Drosophila has previously been implicated in the regulation of cell size and number (Leevers et. al, 1996). Expression of a dominant negative PI3K ($PI3K^{DN}$) construct, which partially blocks endogenous PI3K function (Leevers *et al.*, 1996), caused a rescue of the $Baboon^{QD}$ overgrowth phenotype (Figure 13 C, G). This result indicates that PI3K is genetically downstream of Baboon, as it was able to rescue the phenotype. As well, expression of wtGSK3β caused a partial rescue of the $Baboon^{QD}$ phenotype (Figure 13 D, H). When the $Baboon^{QD}$ construct was expressed with the GMR promoter, as less severe phenotype was seen, resulting in increased organ size and bristle size, with no outgrowth of ommatidia seen from the eye (Figure 14 A, E). Coexpression of $PI3K^{DN}$ also rescued the $Baboon^{QD}$ phenotype, providing additional confirmation of the results noted above (Figure 14 B,F). Expression of either wtPI3K or Akt with $Baboon^{QD}$ caused a synergic effect on growth of the eye, with many more bristles being noted in the eye (Figure 14 C, D, G, H).

We also wanted to extend our understanding of the components of this pathway to establish the roles of dFOXO and Shaggy in modulating the $Baboon^{QD}$ phenotype. The

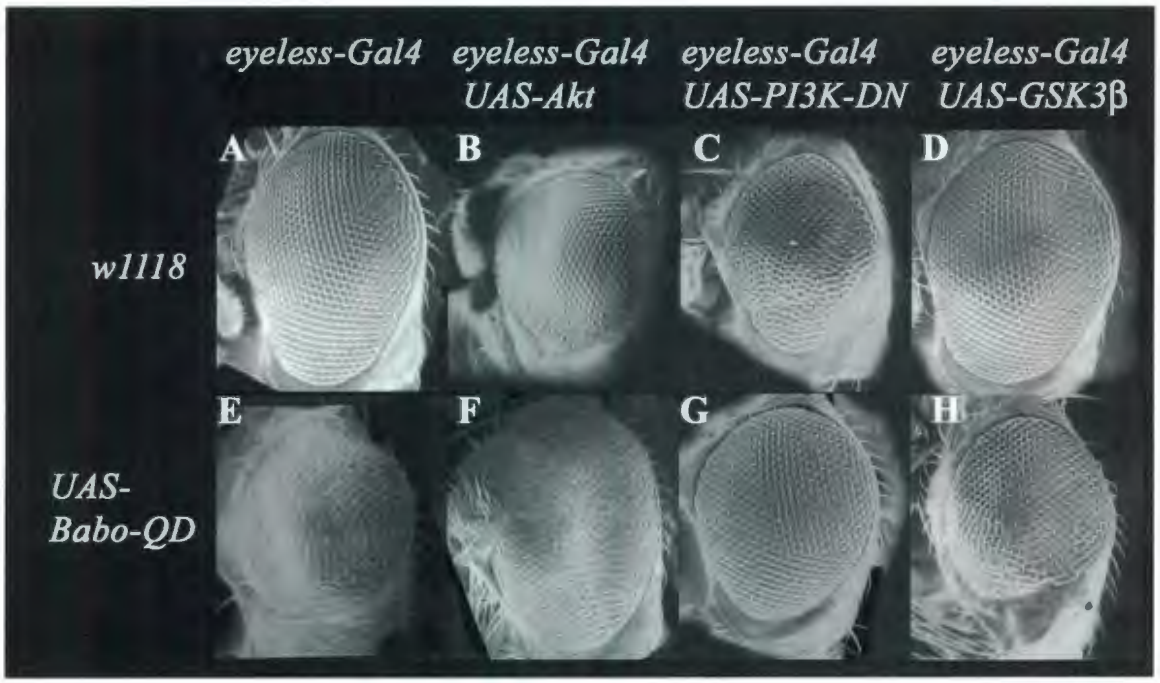

Figure 13: Activated form of TGF-β receptor interacts with PI3K signalling components in Drosophila.

Using the UAS/Gal4 system of ectopic gene expression in Drosophila, we expressed the activated form of the Baboon receptor ($Babo^{QD}$) with PI3K signalling components (Akt, PI3K-DN and GSK-3β) under the control of the *eyeless* promoter. Eyeless-Gal4/UAS-$Babo^{QD}$ reveals a phenotype that is grossly overgrown. Expressing dAkt in conjunction with $Babo^{QD}$ causes increased overgrowth relative to activated Baboon alone, whereas expression of a $PI3K^{DN}$ mediates Baboon overgrowth. Expression of GSK also controls overgrowth of ommatidia, however the rounder eye phenotype of the $Babo^{QD}$ remains. All flies are representative of phenotypes seen, and at least 3 separate animals were examined by SEM. (A) *eyeless-Gal4/+* (B) *eyeless-Gal4; UAS-$PI3K^{DN}$* (C) *eyeless-Gal4; UAS-Akt* (D) *eyeless-Gal4; UAS-wtGSK3β* (E) *eyeless-Gal4/+; UAS-$Babo^{QD}$/+* (F) *UAS-$Babo^{QD}$/eyeless-Gal4; UAS-$PI3K^{DN}$* (G) *UAS-$Babo^{QD}$/eyeless-Gal4; UAS-Akt* (H) *UAS-$Babo^{QD}$/eyeless-Gal4; UAS-wtGSK3β*

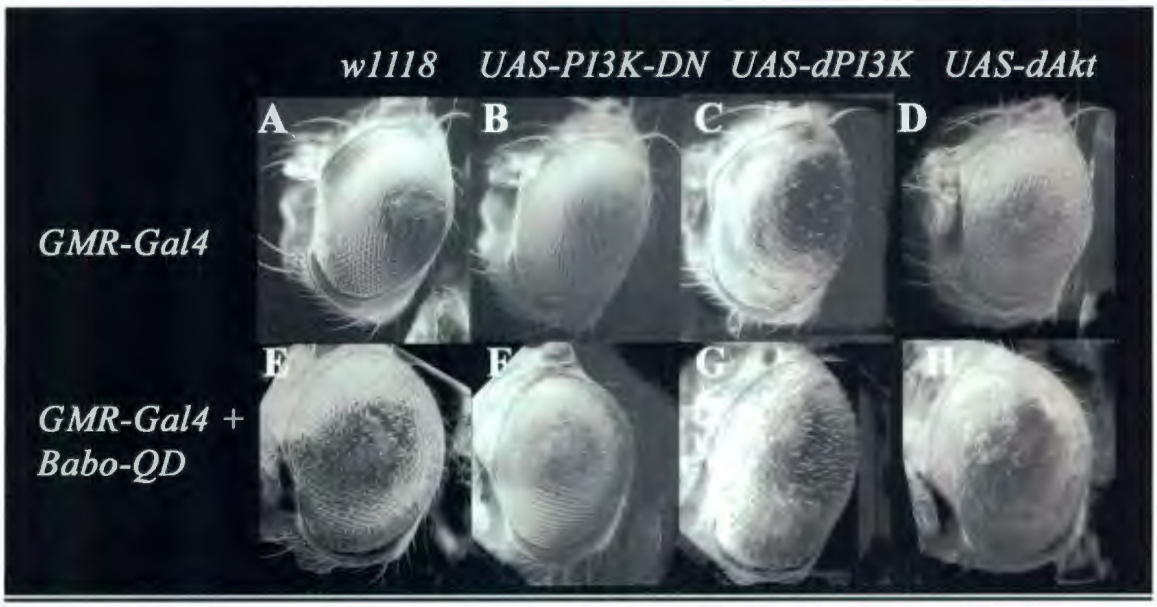

Figure 14: TGF-β Type I receptor interacts with PI3K signalling components in Drosophila.

Using the UAS/Gal4 system of ectopic gene expression in Drosophila, we expressed UAS-Babo^{QD} with PI3K signalling components (PI3K^{DN}, wt-PI3K and dAkt) under the control of the *GMR* promoter. GMR/Activated Baboon reveals a phenotype that is overgrown. Expressing wt-PI3K or Akt in conjunction with Baboon causes increased overgrowth relative to activated Baboon alone, whereas expression of a PI3K^{DN} reduces Baboon overgrowth indicating a genetic link between PI3K and TGF-β receptor signalling. All flies are representative of phenotypes seen, with at least 3 separate animals being examined for SEM. (A) *GMR-GAL4/+* (B) *GMR-Gal4; UAS-PI3K^{DN}* (C) *GMR-Gal4; UAS-dPI3K* (D) *GMR-Gal4; UAS-dAkt* (E) *GMR-Gal4/UAS-Babo^{QD}* (F) *GMR-Gal4; UAS-Babo^{QD}/ UAS-PI3K^{DN}* (G) *GMR-Gal4; UAS-Babo^{QD}/UAS-dPI3K* (H) *GMR-Gal4; UAS-Babo^{QD}/UAS-dAKT*

phenotype of dFOXO when expressed in the eye is the overall organ size is smaller with missing ommatidia and bristles. As describe by Kramer *et al.* (2003) the phenotype of overexpression of mFOXO is similar due to the high level of conservation between mouse Drosophila genes. Misexpression of the Baboon^{QD} transgene with dFOXO or mFOXO caused a rescue of the FOXO phenotype (Figure 15 B,C,F,G) when compared to controls GMR-Gal4 (A) and GMR-Gal4; Babo^{QD} (E), with a rescue of eye size, cell number and bristles as observed by visual observation. This result arises from two possibilities: 1) Baboon rescues dFOXO through an Akt dependent mechanism or 2) Baboon simply increases growth of ommatidia through a FOXO-independent mechanism. To address this, we utilized a form of FOXO (mFOXO^{AA}) that has been previously been shown to be unresponsive to Akt as two of its Akt responsive phosphorylation sites have been mutated to alanine (Kramer *et al.*, 2003) which causes the protein to be 'always on'. mFOXO^{AA} causes a much more severe phenotype than expression of either dFOXO or mFOXO (Figure 15 H compared to B, D). Misexpression of Baboon^{QD} with mFOXO^{AA} (Figure D,H) was unable to rescue the effects of FOXO. This strongly indicates that the rescue of FOXO by Babo is dependent on the Akt phosphorylation sites, placing Babo genetically upstream of both dAkt and dFOXO. Another downstream target of Akt is GSK-3β. Drosophila contains only one GSK homologue, termed Shaggy. Shaggy in Drosophila is best known for its role in Wingless signaling (Parks *et al.*, 1998). However, other roles for this kinase have been described. Mutants in the Shaggy locus (later discovered to be the Drosophila GSK homologue) cause extra bristle formation (Simpson and Cartenet, 1990) in pro-neural clusters in the thorax. Therefore, Shaggy is thought to be essential for selecting the single cell in the

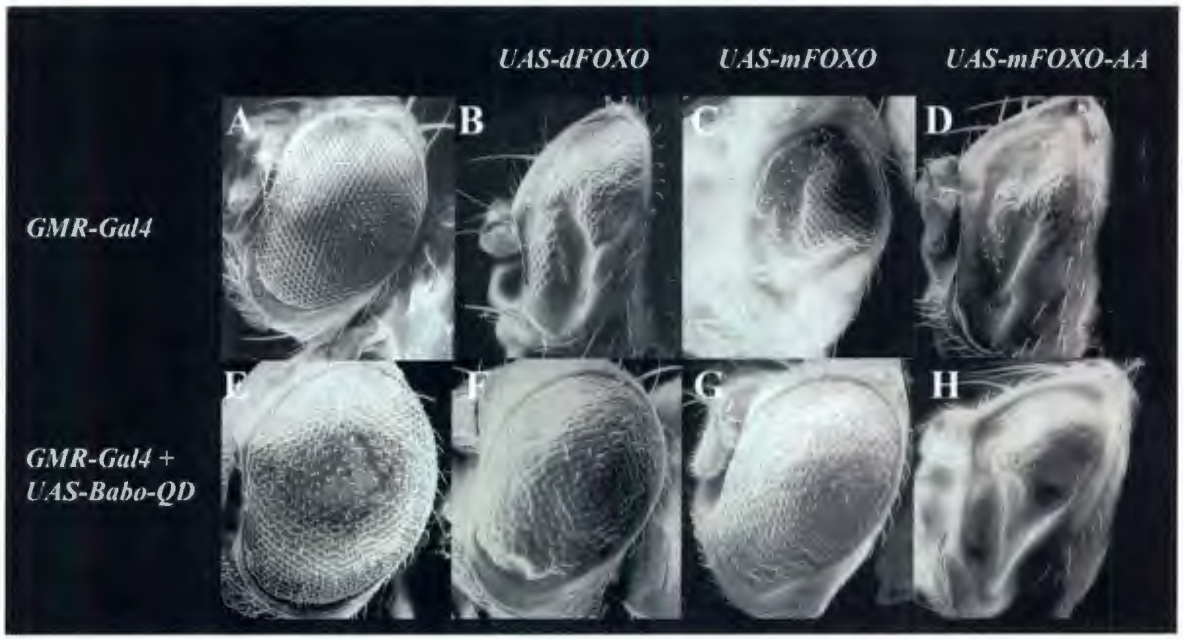

Figure 15: Activated form of TGF-β I receptor interacts with FOXO in Drosophila
Misexpression using the UAS/Gal4 system of dFOXO or mFOXO with the Babo^{QD} transgene caused a rescue of the FOXO phenotype. Misexpression of Babo^{QD} with mFOXO^{AA} did not rescue the phenotype (Figure D,H), with no increases in cell number or bristles. All flies were representative of phenotypes seen, and SEM analysis was done on at least three separate animals. (A) *GMR-GAL4/+* (B) *GMR-Gal4; UAS-dFOXO* (C) *GMR-Gal4; UAS-mFOXO* (D) *GMR-Gal4; UAS-mFOXO^{AA}* (E) *GMR-Gal4/UAS-Babo^{QD}* (F) *GMR-Gal4;UAS-Babo^{QD}/ UAS-dFOXO* (G) *GMR-Gal4 ; UAS-Babo^{QD}/UAS-mFOXO* (H) *GMR-Gal4;UAS-Babo^{QD}/ UAS-mFOXO^{AA}*

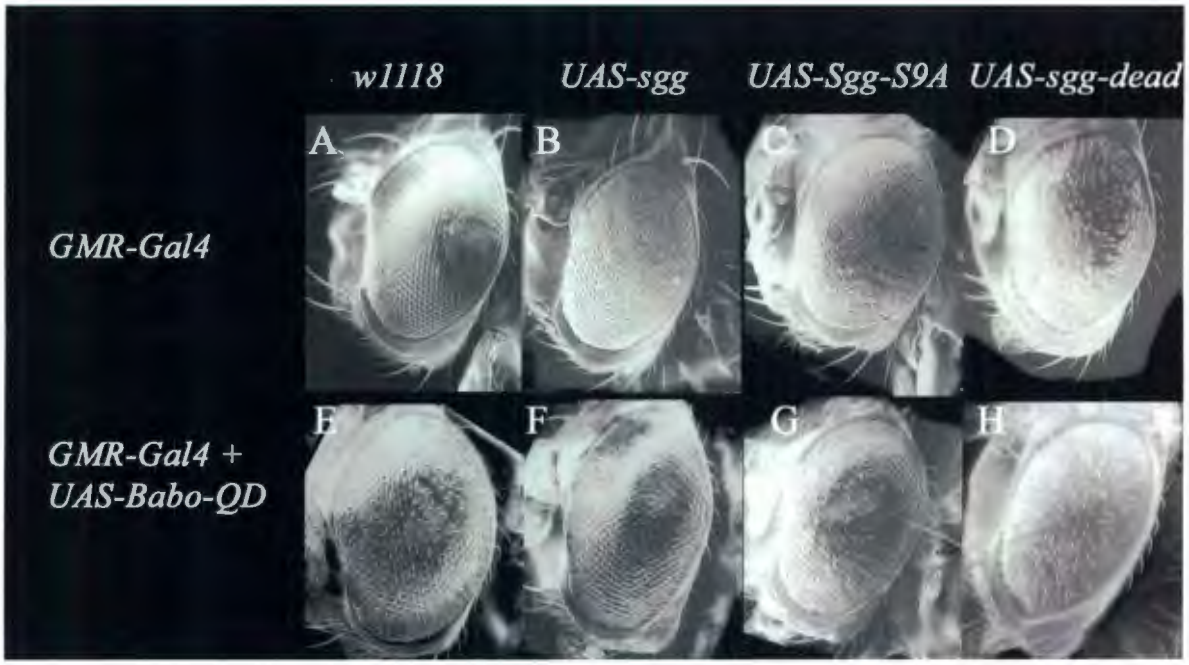

Figure 16: TGF-β Type I receptor interacts with Shaggy signalling components.
We expressed the activated form of the Babo with the Drosophila homologue of GSK, Shaggy (Sgg) under the control of the *GMR* promoter. As shown in Figure 14 the GMR/Activated Baboon phenotype is overgrown. Expressing wt-Sgg, or Sgg that cannot be inactivated by phosphorylation (Sgg^{S9A}), in conjunction with Baboon partially reduces overgrowth. Expression of an Sgg kinase dead reveals much longer and numerous bristles, and coexpression with Baboon worsens the phenotype. This result shows that Babo can signal through GSK/Shaggy in Drosophila. Additionally, the similar and additive phenotypes of Babo and Sgg^{Dead} suggest Babo is one of the signalling components for increased bristle size. (A) *GMR-GAL4/+* (B) *GMR-Gal4; UAS-sgg* (C) *GMR-Gal4; UAS-sgg^{S9A}* (D) *GMR-Gal4; UAS-sgg^{dead}* (E) *GMR-Gal4/UAS-Babo^{QD}* (F) *GMR-Gal4;UAS-Babo^{QD}/ UAS-sgg* (G) *GMR-Gal4 ; UAS-Babo^{QD}/UAS-sgg^{S9A}* (H) *GMR-Gal4;UAS-Babo^{QD}/ UAS-sgg^{dead}*

proneural cluster that will differentiate into bristles. Overexpression of a wt-Sgg or a constitutively active Sgg (Sgg^{S9A}) in the differentiating sensory structure causes a loss of a bristle subtype in the wing and a loss of bristles on the thorax (Bourouis, 2002). No effect of a kinase-dead version of Sgg was observed under these conditions, other than it was unable to repress Wingless signaling.

Expression of Sgg constructs in the eye has not been carefully examined. Given the whole animal work, and the mutant phenotype, we expected to see a bristle phenotype. Expression of Sgg or Sgg^{S9A} under the control of Gal4 had little effect on the eye with perhaps the eye being a little smaller (Figure 16 B, C) whereas expression of Sgg^{dead} revealed longer and numerous bristles when compared to both wildtype (Figure 16A) and the other Sgg constructs. When Sgg is coexpressed with $Babo^{QD}$, suppression of the overgrowth phenotype is seen (Figure 16 B, F). However, coexpression of $Shaggy^{S9A}$ and $Babo^{QD}$ showed an improved rescue over wt-Sgg (Figure 16 C, G compared to B, F). Expression of Sgg^{Dead} and $Babo^{QD}$ had a synergic effect on bristle growth, with a hairy eye phenotype being noted with lots of bristles. Sgg^{dead} also suppressed the $Babo^{QD}$ overgrowth phenotype. This suggests Baboon also normally inactivates Shaggy during eye development to promote growth of ommatidia and is involved in bristle growth/ differentiation.

Chapter 4: Discussion

The area of cellular growth and its relationship to cell cycle is a highly researched area, with relevance to many human disorders such as cancer. Various growth factor pathways have been shown to be involved. One of note is TGF-β signaling; in particular because TGF-β causes distinct responses in different cell types, suggesting this important cytokine has different signalling pathways. The classical Smad signalling pathway is essentially the same in all cell types, leading to the hypothesis that different cell types have additional signalling pathways. Indeed, many previous studies have shown that TGF-β can act in a Smad-independent manner to activate signalling in mesenchymal lineages. TGF-β can activate ERK signalling kinase, along with Rho-like GTPases, Rho A, Rac1 and cdc24 (Edlund, 2002). When activated by TGF-β, each contributes to cytoskeletal reorganization. Recently, TGF-β has been shown to specifically activate PAK2 in fibroblast cell lines but not epithelial cells in a pathway that requires cdc42 (Wilkes *et al.*, 2003). This event was shown to be independent of Smad signaling and dependent on TGF-β receptors. Inhibition of PAK2 leads to defects in TGF-β mediated growth and MT in these fibroblast cell lines. A more recent report (Wilkes *et al.*, 2005) has linked TGF-β receptors to both PI3K and Akt in a linear pathway, however, nothing was reported on signalling downstream of Akt. This report also determined that in epithelial cells, PI3K/Akt pathways are not activated. A role for PI3K has also been described in epithelial to mesynchemal transition (EMT) of epithelial cultures downstream of TGF-β through activation of the PI3K target, Akt (Bakin *et al.*, 2000; Shin *et al.*, 2001). Increased activation of PI3K has been shown in fibroblastic cell lineages, as well as epithelial cultures (Kim *et al.*, 2002). However, especially with

epithelial cultures, several groups fail to show 'activation' of Akt when stimulated with TGF-β (Conery *et al.*, 2004; Seoane *et al.*, 2004; Wilkes *et al.*, 2005). Since the major question appears to be how TGF-β causes growth stimulation versus growth inhibition, the study of PI3K/Akt signalling may be one method by which different cell types interpret TGF-β signalling. This study provides convincing evidence that TGF-β is signalling via PI3K/Akt in both mammalian cell culture and *Drosophila melanogaster* in a cell type specific manner.

4.2 Fibroblast Signalling

Our results indicate that TGF-β activates Akt by phosphorylation at Serine 473, and this event is dependent on a PI3K and other upstream kinases in fibroblasts. Another upstream kinase, PDK1 is also required for Akt phosphorylation at threonine 308. Akt activation, through phosphorylation, also requires intact TGF-β receptors in order for signal propagation. These data agrees with Wilkes *et al.*, (2005) who found a similar trend with respect to Akt phosphorylation at Serine 473 and Threonine 308 increasing until 120 minutes when fibroblast cells were stimulated by TGF-β1. This group found that TGF-β was able to induce PI3K activity in fibroblast lines starting after 120 minutes of stimulation and LY29002 (a PI3K inhibitor) abolished the activity. Akt phosphorylation by TGF-β was also abolished by LY29002 addition and use of a DN-PDK-1.

Wilkes *et al.*, (2005) also reported that Akt phosphorylation in fibroblast cells is: 1) a SMAD-independent event and 2) through the use of inhibitors of either transcription or translation that TGF-β mediated phosphorylation of Akt is independent of these processes. . This suggests that phosphorylation of Akt by TGF-β is a direct event, not a

result of SMAD-mediated transcription. Our data characterized further downstream targets of Akt, such as GSK-3β in fibroblasts. I see increased phosphorylation of GSK-3β over time in fibroblasts, and this event is dependent on PI3K and Akt. This data suggests that TGF-β has the ability to directly inactivate GSK-3β, through S9 phosphorylation via a direct linear pathway. Given that inactivating Cyclin D1 along with other targets involves GSK-3β in inhibiting the cell cycle, inactivation of the GSK-3β kinase activity should have a direct effect of increasing progression through cell cycle. This inactivation of GSK-3β should also increase growth as increased progression of cell cycle is directly linked to cellular growth. In this study I show TGF-β stimulation results in less Cyclin D1 phosphorylation, previously been shown to be GSK-3β dependent (Diehl *et al.*, 1998) and increased nuclear localization of Cyclin D1 and complex formation with CDK-4. All of these events have been correlated to increased G1/S progression in various cell lines, including NIH-3T3 (Ewen *et al.*, 1993). As well, our data shows that Akt inhibition leads to decreased growth in NIH-3T3 fibroblast cells. This result consequently provides some evidence that TGF-β can control the cell cycle directly through a signalling cascade that does not depend on Smad signalling. Other groups have reported PI3K pathways acting on cell cycle by interactions with Smads (Seaone *et al.*, 2004). The current theory is that different Smad binding partners associating with the promoters of genes cause cell specific differences, and TGF-β does not signal to other pathways directly from the receptors. However, our data, in addition to that published by Dr. Leof's group (Wilkes *et al.*, 2005; Wilkes and Leof, 2006) suggests that there are other pathways directly involved in TGF-β signalling. To my knowledge, this is also the first report of TGF-β having a direct effect at the protein level on regulation of cell cycle components.

Use of our Akt IV inhibitor also prevents TGF-β mediated morphological transformation and growth of fibroblasts. Other targets of the TGF-β receptor complex such as PI3K and PAK-2 have also been shown to be involved in the MT and growth of fibroblasts (Wilkes *et al.*, 2003; Wilkes *et al.*, 2005). Akt has previously been shown to affect growth and cell cycle in different contexts using many targets. In breast cancer tumours, Akt can phosphorylate $p27^{kip1}$ to promote cytosolic localization (Liang *et al.*, 2002) thereby leading to increased G1 progression. Negative regulation of FOXO by Akt results in decreased transcription of Cyclin D1 (Schmidt *et al.*, 2002) and enhanced transcription of $p27^{kip1}$. Akt also negatively regulates GSK-3β, causing decreased phosphorylation of Cyclin D1, resulting in nuclear localization and higher protein levels (Diehl *et al.*, 1998). Overexpression of Akt in both mice (Shioi *et al.*, 2002) and Drosophila (Verdu *et al.*, 1999) results in larger total organ size, with increases in cell size observed. Knockout mice in Akt 1/2 and Drosophila *akt* mutants both display severe reduction in body size (Peng *et al.*, 2003; Staveley *et al.*, 1998). All these reports illustrate the critical role of Akt in G1/S cell cycle progression and the overall growth of cells. In light of the above data, TGF-β can activate Akt, which then inactivates GSK-3β leading to increased levels of Cyclin D1 should cause increased G1/S progression and increased cellular growth. My data also illustrates that Akt is probably necessary for TGF-β mediated growth in fibroblasts (Figures 6 and 8).

Overall, in fibroblasts, I show that TGF-β stimulates Akt activation. This leads to GSK-3β inactivation and a decrease in Cyclin D1 phosphorylation coupled to increased protein levels and nuclear localization. These events are correlated with increased progression into S phase, leading to increased rates of cell division. Indeed, blocking Akt

activation prevents TGF-β growth stimulation of fibroblasts, GSK inactivation and Cyclin D1/CDK4 complex formation, suggesting that the Akt signalling is needed for this process.

4.3 Epithelial Signalling

As well as characterizing a fibroblast-signalling pathway, I wished to determine if this PI3K/Akt signalling pathway could differentiate fibroblasts from epithelial cells. A role for PI3K has also been described in EMT of epithelial cultures downstream of TGF-β and in preventing apoptosis of epithelial cultures through activation of the PI3K target, Akt (Bakin *et al.*, 2000; Ju *et al.*, 2005). Increased activation of PI3K has been shown in mesenchymal cell lineages as well as epithelial cultures (Kim *et al.*, 2002; Shin *et al.*, 2001). However, with epithelial cultures, several groups failed to see "activation" of Akt when stimulated with TGF-β (Seaone *et al.*, 2004; Wilkes *et al.*, 2005). Epithelial cells, in contrast to fibroblasts, are growth-inhibited by TGF-β (Laiho *et al.*, 1990). Therefore, I expected this signalling pathway not to be activated in epithelial cells, similar to the results obtained by Wilkes *et al.* (2005). Surprisingly, I found that TGF-β caused a decrease in both Akt phosphorylation and that of the upstream kinase, PDK-1. Decreases in the phosphorylation of Akt have been shown to correlate with loss of kinase activity (Brazil *et al.*, 2001). In contrast to fibroblasts, GSK-3β phosphorylation was likewise decreased in response to TGF-β, which corresponds to an increase in GSK-3β activity (Cross *et al.*, 1995). Increases in GSK-3β activity should lead to increased phosphorylation of its downstream targets, including Cyclin D1. As expected, I saw decreased protein levels of Cyclin D1 and a decreased association of Cyclin D1 with its binding partner CDK-4. These events have been shown to be associated with a G1/S

arrest (Diehl *et al.*, 1998), indicating that TGF-β stimulation of epithelial cells causes a decrease in cell cycle proteins. This also suggests this may be one of the mechanisms by which TGF-β can halt the cell cycle in epithelial cells, independent of Smad signalling. A possible model is that TGF-β causes deactivation of Akt, making epithelial cells quiescent, and that Smad signalling is necessary for sustained maintenance of the cell cycle arrest program through increased transcription of p27 and other cell cycle inhibitory factors such as p21 (Laiho *et al.*, 1990).

Thus far, the data described indicates that TGF-β signalling through Akt is one pathway that differentiates fibroblast cells from epithelial cells. This novel pathway may help define how TGF-β can cause one cell type to grow and another to halt growth. As well, in certain epithelial-derived cancer cells resistance to the anti-growth effects of TGF-β has been shown (Reviewed in Massague, 2000; Elliot and Blobe, 2005) and in some cases these cells even respond by increasing growth. This pathway may also be key to EMT, a biological process where an epithelial cell acquires characteristics similar to fibroblasts. EMT is thought to be a defining event in the development of epithelial cancer metastasis, and TGF-β may mediate this event even in normal epithelial cells (Elliot and Blobe, 2005). However, normal cells revert to normal morphology with withdrawal of TGF-β while in carcinoma cells remain transformed. The activation of Akt under inappropriate circumstances may explain why such events happen, whereby the epithelial cells begin to take on a fibroblast-like phenotype. Overactive Akt may lead to the cells becoming resistant to TGF-β growth inhibitory signals, causing the cells to behave like a mesenchymal cell type.

Our data in epithelial cells conflicts with previous reports that either 1) show an increase in Akt activation in response to TGF-β1 stimulation (Yan *et al.*, 2000; Shin *et al.*, 2001; Jun *et al.*, 2005) or 2) no change in Akt activation (Seaone *et al.*, 2004; Wilkes *et al.*, 2005). However, with respect to the 'Akt activation', the cell lines used were mostly cancerous lines, unlike the normal lines used here. Overexpression of normal Akt is observed at high frequency in the epithelial cancers such as pancreatic, ovarian and breast cancer (Bellacosta *et al.*, 1995) and targeting Akt to the membrane can induce carcinogenesis in normal cells (Aoki *et al.*, 1998). Therefore, the cell lines used may already have higher levels of Akt, and be unresponsive to TGF-β inhibition. In addition, Akt was usually overexpressed, unlike our study, which used the endogenous protein. Overexpression of Akt may mimic a fibroblast or cancer like state in epithelial cells, where they do not respond normally to TGF-β stimulation. Wilkes *et al.*, (2005) describe no changes in Akt activation following TGF-β1 stimulation, however the cells in question were starved in low serum conditions and plated at a much higher density than this study. At the beginning of the thesis work, I undertook studies to determine the effect of cell density, and found more consistent results from plating a lower cell density (~50% confluence versus 90-100% confluence). Cell density is an important determinant in cell growth, given that the cell lines used here display contact inhibition. Therefore, plating at higher densities may halt cell cycle progression and growth at the time of the assay. With regards to the low serum condition, if you were unable to detect a basal level of Akt phosphorylation (*i.e.* the cells have become quiescent), it would be difficult to determine the effect TGF-β may be having on this signalling system. As well, epithelial cells respond to TGF-β by going into G1/S arrest. If the cells are quiescent (by both contact

inhibition and serum depravation), short-term responses to TGF-β may be significantly reduced. Also, contact with other cells may increase responses to intergin/ILK- linked signalling either TGF-β related or not (Wu and Dedhar, 2001). Given that ILK is a potential Serine 473 kinase for Akt (Delcommenne *et al.*, 1998), an interesting avenue of research could be the relationship between cell density and activation of Akt through an intergin and TGF-β linked mechanism.

Chapter 4.4 Drosophila Model of TGF-β/Akt signaling

One of the major questions left to be answered is whether this is a pathway for growth *in vivo*. To address these questions, I used the model system Drosophila to attempt to determine a genetic interaction between the TGF-β type I homologue (Baboon) and PI3K signalling components. *Babo* mutants display a striking phenotype, in that they do not affect patterning, but instead influence cell proliferation in the developing larvae (Brummel *et al.*, 1999). *Babo* mutants have smaller brains and correspondingly smaller imaginal discs, with no signs of apoptosis but reduced cell proliferation. Since *babo* mutants die before adulthood, clonal analysis of the adult wing was undertaken, which results in smaller wings. An activated form of *babo* ($Babo^{QD}$) was expressed in the developing wing disc, which resulted in a 30% increase in organ size. Few other molecules in Drosophila have the ability to decrease imaginal disc size without patterning defects, with PI3K/Akt being another example (Leevers *et al.*, 1996; Verdu *et al.*, 1999).

Given the above phenotypes of smaller eye imaginal discs in *babo* mutants, I choose the developing eye as a system to study potential Baboon/PI3K interactions. The Drosophila eye is a highly organized and repetitive structure made of individual cells

called ommatidia. Ommatidial clusters are generated by a recruitment of cells behind the morphogenetic furrow and undergo dramatic growth and differentiation during larval and pupal stages to generate the adult eye (Baker, 2000). The eye also is an excellent system to study cellular growth decisions. When I expressed the normal Baboon receptor in the developing eye using both early and late drivers (*eyeless-Gal4* and *GMR-Gal4*), it had little effect. This result is consistent with Brummel *et al.* (1999) who saw little effect of overexpression of the normal receptor. I also misexpressed a DN-dActivin receptor, as dActivin is an endogenous ligand of Babo, and saw no effect on cellular growth. Although this may appear surprising, however another TGF–β ligand for Babo has since been found, dawdle (Parker *et al.,* 2006). Dawdle therefore may signal upstream of babo to mediate some of its effects.

To create an activated system, I used the previously characterized $Babo^{QD}$ receptor. Using two different drivers (*eyeless-Gal4* and *GMR-Gal4*), I found that $Babo^{QD}$ caused overgrowth of the eye. Co-expression of a $PI3K^{DN}$ with the $Babo^{QD}$ receptor caused a rescue of this phenotype, and overexpression of normal PI3K/Akt caused a synergic increase in growth. As well as an overgrowth phenotype, I found an increase in the size of the bristles in the eye. Coexpression with either dAkt or PI3K was additive with this phenotype. This suggests that $Baboon^{QD}$ can signal to dAkt or dPI3K and by blocking signals through dPI3K, a loss of signalling results. Therefore, Akt and PI3K act *in vivo* in the developing eye in a downstream pathway to a TGF-β receptor, Baboon.

I also characterized the Drosophila dFOXO and GSK-3β homologues with respect to Baboon signalling. Coexpression of either mFOXO or dFOXO with $Baboon^{QD}$ resulted in a rescue, while coexpression with $mFOXO^{AA}$ resulted in little rescue of the FOXO

phenotype. This result indicates Babo rescues dFOXO through an Akt dependent mechanism. It also places Babo genetically upstream of dFOXO. These results agree with those of Kramer *et al.* (2003), where expression of PI3K and Akt were also unable to rescue $mFOXO^{AA}$ phenotype. This again, strongly implies that dFOXO is a target of Babo activity in the developing eye.

There is also strong genetic evidence from *C. elegans* for a TGF-β/FOXO pathway. DAF-7 (TGF-β like ligand) is involved in dauer formation in *C. elegans* (Riddle and Albert, 1997). Dauer is a specialized state that L1 larvae can enter upon food withdrawal or environmental stresses to help the worm survive. Another dauer pathway in *C. elegans* is the daf-2 /daf-16 pathway that is also implicated in longevity regulation. Daf-2 encodes insulin-like peptide and Daf-16 is the worm FOXO homologue. Both pathways are thought to mediate dauer arrest as separate arms. However, Runkvun's group in 2001 demonstrated that DAF-7 could mediate DAF-16 nuclear relocalization at the L2 larval transition stage (Lee *et al.,* 2001). This was thought to be an indirect interaction. However, a recent report, implicates DAF-7 and insulin-like pathways more directly (Shaw *et al.*, 2007). A microarray analysis of DAF-7 and insulin pathway transcriptional outputs indicated a high level of similarity between the two profiles. Analysis of DOD (Downstream of DAF-16) sites revealed a high level of DAF-16 regulated genes downstream of DAF-7 signalling. DAF-16 was also required for DAF-7 - mediated increases in longevity, indicating it is genetically downstream of DAF-7. Finally, DAF-7 regulates DAF-16 nuclear relocalization and increases SOD-3,a target of DAF-16 transcription. The authors of this paper again suggest that the effects are indirect, however coupled with the evidence presented here, it seems likely that at least some

TGF-β-mediated responses signal directly to FOXO in a whole animal. dFOXO displays a 'wandering larvae' phenotype and an inability to sense food that is thought to be analogous to the dauer state of *C. elegans* (Kramer *et al.*, 2003). Further research could be directed into dissecting if the role of DAF-7 on DAF-16 is through Akt-dependent phosphorylation. *Babo* mutants could also be examined for sensitivity to nutritional challenges, and if this influences signaling to dFOXO.

In addition to dFOXO, we also wished to see if the GSK homologue, Shaggy could ameliorate the Babo phenotype. Overexpression of Shaggy with $Babo^{QD}$ gave a partial rescue of the overgrowth phenotype. A form of Sgg that cannot be phosphorylated by Akt, Sgg^{S9A} provided more of a rescue than overexpression of the normal Sgg. Of special interest is the $Shaggy^{Dead}$ construct, which showed an additive phenotype with respect to bristles, but not with the growth phenotypes. This suggests the kinase activity of Shaggy is needed for normal bristle outgrowth, but not suppression of TGF-β-mediated growth.

Overall, the Drosophila results provide evidence that TGF-β and PI3K/Akt signal together *in vivo* in the control of cellular growth. TGF-β is normally cytostatic to neuronal cell lineages, however the role of Babo seems to be to promote cell proliferation in neuronal lineages. Activins however promote neurogenesis, and as Babo is closely related to both ligand families, it would appear that this Type I receptor causes a similar phenotype. However, as TGF-β are also involved in late organogenesis, similar to Babo, it would be interesting to dissect out the role of TGF-β signaling to Akt in both developmental and differentiated contexts.

4.5 Conclusions and future directions

Since TGF-β causes fibroblasts to grow, increased phosphorylation of Akt and GSK-3β represents another mechanism of growth control in conjunction to the previously characterized Smad pathway. To our knowledge, this is one of the first reports of TGF-β directly regulating cell cycle components, independent of RNA synthesis. As well, the Drosophila TGF-β Type I receptor, Baboon, interacts with PI3K/Akt signalling. A lesser interaction is seen with overexpression of GSK, suggesting that there may be other targets of PI3K/Akt involved in TGF-β mediated growth control *in vivo*, such as FOXO. FOXO has also been implicated as a transcriptional binding partner for Smads (Seaone *et al.*, 2004), allowing for integration of multiple TGF-β pathways. The activation of Akt in fibroblast, and the inhibition in epithelia by TGF-β provide a mechanism for cell type specific response to growth. These results also provide a possible alternative mechanism for loss of growth control by TGF-β in cancer, with epithelial cells potentially activating Akt and causing a lack of growth inhibition.

However, this work raises several interesting questions. One is how the signal gets from the TGF-β receptors to PI3K. Several possibilities could exist, the simplest being that PI3K binds directly to the TGF-β receptor. Experimentally, one could use wildtype and mutant Type I receptor (L45 loop defective) to see if PI3K can bind and transduce the signal. Previous research using an L45 loop defective mutant of Alk-5 was able to retain JNK activity, while losing Smad activity (Itoh *et al.*, 2003). If PI3K binds mutant Type I, then this would possibly indicate the signalling in fibroblasts is truly Smad-independent. The converse experiment could be carried out in epithelial cells, with wildtype Type I receptor in an attempt to see if they do bind at all.

As previously discussed in the introduction, this is unlikely it has already been suggested that an adaptor protein is probably needed (Yi *et al.*, 2005). However, a serious conceptual issue is that TGF-β receptors do not have any phosphorylated tyrosines for a PTB adaptor protein to bind to. A tantalizing report by Galliher and Schiemann, 2007 demonstrates that the tyrosine kinase Src can phosphorylate TGF-β Type II receptors on Y284. They also demonstrate that this residue is essential for Grb2 binding, a PTB adaptor protein. TGF-β Type II receptors lacking this site were unable to activate p38 Mapk and bind Grb2. However, other groups have already suggested that the Type I receptor kinase activity is required for PI3K kinase activity (Yi *et al.*, 2005, Wilkes *et al.*, 2006) and indeed the result generated in this study also suggest that both Type II and Type I kinase activity is needed for Akt activation. However, the TGF-β Type I Receptor has recently been shown to be a dual specificity kinase, with the ability to directly phosphorylate ShcA on both tyrosine and serine residues (Lee *et al.*, 2007). This tyrosine kinase activity could help explain recruit of PI3K to the TGF-β receptors by adaptor proteins such as ShcA. The authors also demonstrate that both receptors are needed for ShcA phosphorylation, which leads to Grb2/Sos recruitment and downstream Erk activation. However, a major issue is how ShcA is recruited to the Type I receptor when there is no apparent phosphorylated tyrosines, and the PTB domain of ShcA was thought to be essential for the interaction. The tyrosine 424 of the Type II receptor is highly conserved with Type I receptor and forms a potential PTB binding motif. Performing site directed mutagenesis on these sites in both the Type I and II receptors, and seeing if this 1) eliminates Shc recruitment and phosphorylation and 2) eliminates PI3K activation and

Akt phosphorylation in fibroblasts would allow one to see if recruitment of adaptor proteins is truly essential in activation of PI3K.

An additional potential 'adaptor' for the TGF-β receptors is ErbB3, a EGF receptor that can bind ligand, but is unable to convey the signal into the cell directly as it lacks a kinase domain. Another recent report demonstrates that TGF-β can increase association of ErbB3 with the p85 subunit of PI3K, which is assumed to be caused indirectly by TGF-β inducing phosphorylation of another regulatory protein, TACE (Wang *et al.*, 2008). This occurred in cancerous epithelial cells, so it would be interesting to see if a similar trend happens in 'normal' cell lines, and if not, this could be a key regulatory component to the loss of cytostatic growth control in cancerous epithelial cells.

Another possibility is that there are other signalling components acting between PI3K and Akt. In our lab, we have shown that the ILK kinase activity on Akt is upregulated in response to TGF-β stimulation in fibroblasts. The activation of the ILK kinase could explain many of the cell density effects seen in this work, such that tightly packed cells have increased Akt signalling through the activation of intergrins. To test the dependence of ILK in TGF-β/Akt signalling one could employ gene knockdown techniques such as RNAi or morpholinos to attempt to assess ILK functions. Another approach would be to further clarify the target the Akt IV inhibitor acts upon, as it does act on the upstream Serine 473 kinase.

Other potential input into these signalling pathways is PTEN. I have done preliminary blots that show an increase in phosphorylation of PTEN in fibroblasts and a decrease of phosphorylation in epithelial cells. Phosphorylation of PTEN inactivates the

protein's activity (Torres and Pulido, 2001), which would result in less phosphatase activity at the inositol phospholipids produced by PI3K. Increasing PTEN phosphorylation would result in more phosphatase activity in epithelial cells, indirectly leading to decreased Akt phosphorylation. This would also help explain how Akt is actively dephosphorylated by TGF-β stimulation in epithelial cells.

One potential issue with this theory is how the signal gets transduced from the TGF-β receptors to PTEN. PP2A is a potential cofactor of the TGF-β receptors (Petritsich *et al.*, 2000), and can mediate G1 arrest in epithelial cells. PP2A can also dephosphorylate PTEN, providing a potential method of regulation in epithelial cells. One potential way of addressing this question would be the use of PP2A inhibitors to see if this could block TGF-β effects on PTEN and Akt phosphorylation. PTEN phosphorylation may be potentially regulated by PP2A, providing a possible link between the TGF-β receptor and down regulation of Akt signaling in epithelial cells.

Other possibilities for epithelial cells are that the trends noted in this thesis are not truly Smad-independent. A direct way to test this would be to use the L45 loop mutant ALK-5 to see if the Akt dephosphorylation is seen in the absence of Smads. Also, there are several Smad knockout mice that could be combined to generate a Smad 2/3 null line that would avoid the artifacts of overexpression that might be seen with using the receptor lines. Use of inhibitors of translation and transcription could also add in dissecting out if the epithelia phenomena are truly independent of Smad transcription.

If the data in epithelial cells is Smad-dependent, one potential method of regulation could be through the lipid phosphatase SHIP (Itoh *et al.*, 2007). Smads have previously been implicated in influencing SHIP levels. Since SHIP takes the P3

phosphate off the PI3 lipids, this may lead to inhibition of Akt phosphorylation. This may also explain the 'delay' in signalling seen in epithelial cells versus fibroblasts (120 minutes versus 90 minutes), as the events may rely on transcription.

Downstream of Akt, the FOXO transcription factors have not been fully studied in fibroblasts versus epithelial cells. There is already evidence (Seaone et. al, 2004) that FOXO is involved in TGF-β mediated arrest of the cell cycle via interactions with Smads. Since Akt is clearly differentially regulated in fibroblasts versus epithelial cells, this could provide an additional target of Akt to help explain growth regulation. Also, the results from the Drosophila work hint that dFOXO is a more critical target of dAkt activity than GSK, with respect to the growth of the developing eye.

Finally, it remains to be seen if TGF-β-mediated signalling through Akt is perturbed in epithelial derived cancers, such as breast cancer. Repeating some of the experiments outlined in this thesis in breast cancer cell lines of varying metastastic ability and correlating it to Akt activation could shed light upon this question.

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