

A Novel Role for SLK in Transforming Growth Factor-Beta-Mediated Epithelial-to-Mesenchymal Transition

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Abstract

In the late stages of cancer, tumors acquire the ability to spread throughout the body and invade distant tissues in a process called metastasis. Studies have shown that metastasis is responsible for 90% of all cancer-related deaths, making this an important field of study. In breast cancers, 30% of patients overexpress the HER2 oncoprotein, causing a more invasive and metastatic disease. Invasion can be stimulated *in vitro* using the soluble ligand transforming growth factor- β (TGF β) to induce a process called EMT (epithelial-to-mesenchymal transition), where epithelial cells transition into a migratory phenotype through cell-cell junction breakdown. SLK is a Ste20-like kinase that has been linked to many processes, including cell migration and signaling downstream of the HER2 receptor complex. Here we show that the cellular migration and invasion of TGF β -treated normal mammary epithelial cells is significantly impaired in the absence of SLK. Additionally, immunofluorescence analyses demonstrate that SLK knockdown conditions decrease a cell's ability to progress through EMT due to the visible staining of epithelial markers. We find that SLK-depleted cultures express significantly lower levels of Snail1, and fibronectin mRNA levels following TGF- β treatment. Surprisingly, our data demonstrates that SLK kinase activity is not activated downstream of TGF- β stimulation, and that a kinase-dead SLK rescues Snail1 mRNA expression levels. Together these data suggest that SLK plays a novel role in TGF β -induced epithelial-to-mesenchymal transition in a kinase activity-independent manner.

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List of Abbreviations

bHLH	Basic helix-loop-helix
BM	Basement membrane
BMP	Bone morphogenetic protein
Cdc42	Cell division control protein 42
cPML	Cytoplasmic form of promyelocytic leukemia protein
CTBP	C-terminal binding protein 1
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-related kinase
GDF	Growth differentiation factor
GDNF	Glial cell-line-derived neurotrophic factor
Grb2	Growth factor receptor-bound protein 2
GTPases	Guanosine triphosphatase
HER2	Human epidermal growth factor receptor-2
HGF	Hepatocyte growth factor
HMGA2	High mobility group A2
HRG	Heregulin
Ids	Inhibitor of DNA binding

FA	Focal adhesion
FAK	Focal adhesion kinase
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal-to-epithelial transition
MMP	Matrix metalloproteinase
MIS	Mullerian inhibiting substance
PBS	Phosphate buffered saline
PCAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3-kinase
PVDF	Polyvinylidene fluoride
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated-coiled-coil-containing protein kinase
SARA	SMAD anchor for receptor activation
Shc2	Src homology domain containing 2
SLK	Ste20-like kinase
Smurf	Smad-specific E3 ubiquitin protein ligase
Sos	Son of sevenless
TNF- α	Tumor necrosis factor- α
T β RI	Transforming growth factor- β receptor I
T β RII	Transforming growth factor- β receptor II
TGF β	Transforming growth factor- β
TJ	Tight junction

ZEB Zinc-finger E-box-binding homeobox

ZO-1 Zona occludens-1

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1.0 INTRODUCTION

1.1 Ste20-like Kinase

1.1.1 Structure and Function

The Ste20 family of serine/threonine protein kinases are important for both cellular migration and proliferation (Davis, 1993) (Fanger, Gerwins, Widmann, Jarpe, & Johnson, 1997). Well studied in yeast, these kinases are associated with the MAPK pathways and G-protein coupled receptors (Zhao, Leung, Manser, & Lim, 1995). The Ste20-like kinases (SLKs) share homology with the Ste20 proteins and can be further subdivided into three major categories: germinal centre kinases (GCKs), p21-activated kinases (PAKs) and pleckstrin-homology domain-containing PAKs (PH-PAKs) (Sells & Chernoff, 1997). Ste20-like kinase (SLK) was first isolated from guinea pig liver (Itoh, Kameda, Yamada, Tsujikawa, & Kohama, 1997) and is a GCK-family kinase with homologs in both mice (Sabourin & Rudnicki, 1999) and humans (Yamada, Tsujikawa, Itoh, & Kameda, 2000). SLK is associated with a wide range of processes, including cell migration, apoptosis, HER2 signaling, the cell cycle, and embryonic development (Al-Zahrani, et al., 2014) (reviewed in Al-Zahrani, Baron, & Sabourin, 2013). The protein structure of SLK contains three domains: an N-terminal Ste20 kinase domain (amino acids 1-338), a coiled coil region (amino acids 339-788), and a C-terminal disorganized **AT1-46** homology (ATH) domain (amino acids 789-1202) (**Figure 1**). The Ste20 kinase sequence found in all Ste20-like kinases is defined by TPYWMAPE. This sequence is located in the N-terminal domain of SLK (Sabourin & Rudnicki, 1999; Sabourin, Tamai, Seale, Wagner, & Rudnicki, 2000). The activity of SLK is believed to function through a homodimerization event in the *trans* orientation that allows SLK to autophosphorylate and subsequently phosphorylate downstream substrates (Delarosa, Guillemette, Papillon, Han, Kristof, & Cybulsky, 2011; Pike, et al., 2008).

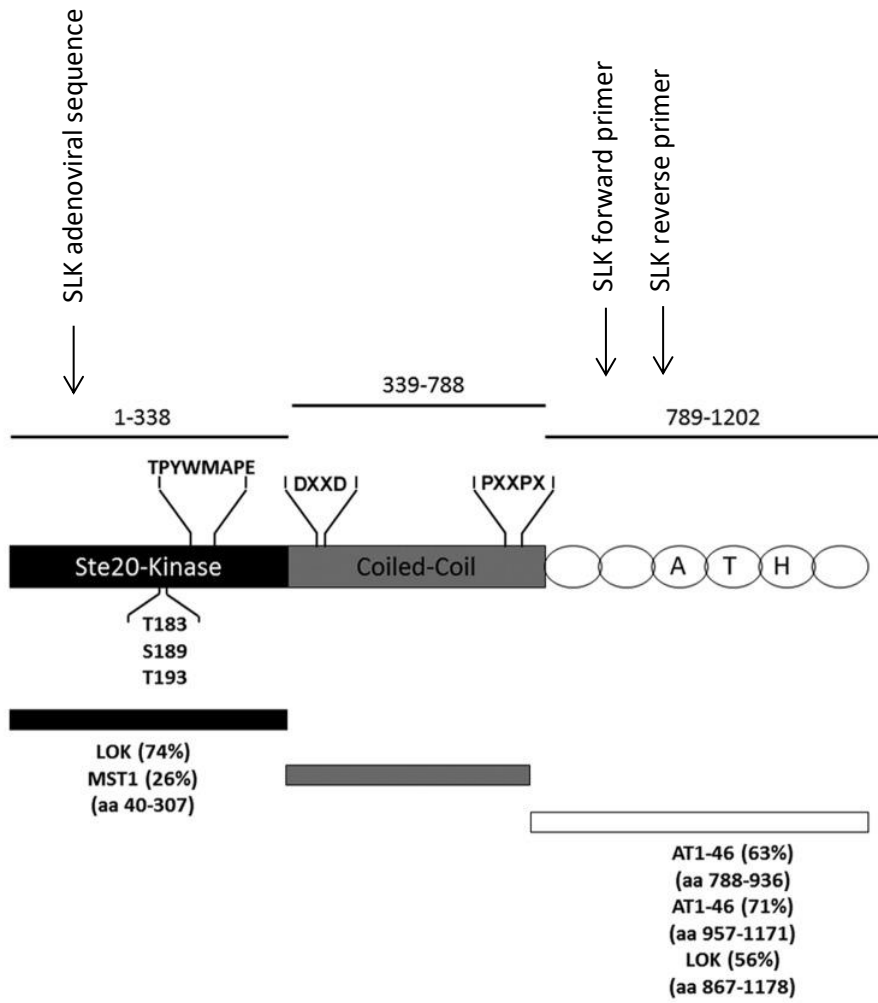


Figure 1. Protein structure and key residues of the Ste20-like kinase. The kinase domain of SLK runs from amino acid residue 1-338 and contains the kinase sequence TPYWMAPE. Three sites thought to be important for kinase activity (T183, S189 and T193). The coiled-coil region of SLK (residues 339-788) is of relatively unknown function but contains a putative consensus SH3 binding site, PXXPX, and a putative consensus caspase 3 cleavage site, DXXD. The disorganized ATH region contains amino acids 789-1202 whose role is not yet well understood. Figure adapted with permission from: (Al-Zahrani, Baron, & Sabourin, 2013).

Previous studies from our lab have demonstrated that a point mutation in full-length SLK at position 63 from a lysine to an arginine residue (K63R) renders the protein kinase-dead (Sabourin, Tamai, Seale, Wagner, & Rudnicki, 2000). Current research aims to clarify SLK activation and establish downstream substrates of the kinase.

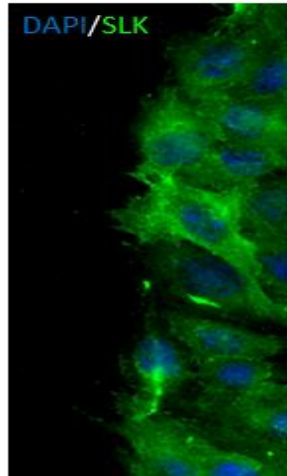
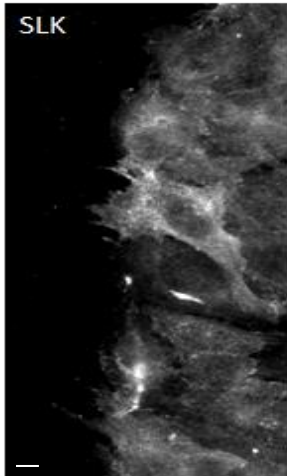
1.1.2 Role in Cell Migration

SLK protein is cytosolic with some reactivity at the cell periphery, co-localizing with lamellipodial markers, tight junction proteins, and the microtubule network (Quizí, et al., 2013). Research from our lab has demonstrated an important role for SLK in adhesion dynamics due to its interaction with microtubules that leads to a reorganization of the cytoskeletal network in cellular migration (Wagner, Flood, O'Reilly, Hume, & Sabourin, 2002). SLK has also been shown to activate downstream of FAK/c-src signaling, demonstrating a key role in focal adhesion turnover (Wagner, et al., 2008). In addition, the kinase activity of SLK is increased upon scratch-wounding and SLK knockdown inhibits cell motility, both supporting a role for SLK in cell migration (Wagner, et al., 2008). Furthermore, SLK phosphorylates paxillin on serine 250 downstream of FAK signaling, which is a critical step in focal adhesion turnover in cellular migration. This event allows for the recruitment of paxillin to the focal adhesion complex (Quizí, et al., 2013). Our lab has also demonstrated that SLK is recruited to the leading edge of migratory cells when scratch-wounded (**Figure 2**). However, there is a subset of SLK that remains cytoplasmic, allowing for the possibility of other potential roles separate from migration.

1.1.3 Role in other signaling pathways

Overexpression of the HER2 oncoprotein in humans (ErbB2 in mouse and Neu in Rat) is linked to an increase in the metastatic and invasive potential of breast cancer tumors

0 min post wounding



60 min post wounding

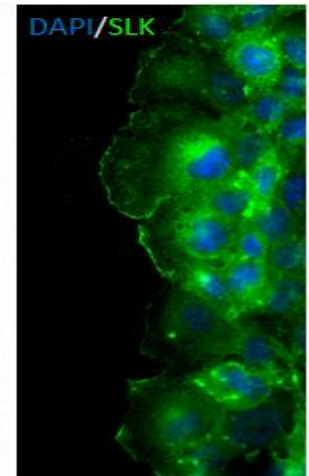
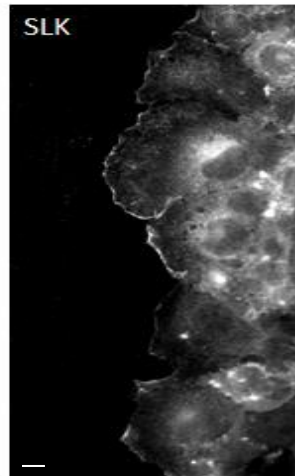


Figure 2. SLK localizes to the leading edge of scratch-wounded cells. Fibroblasts were grown to confluency on a coverslip. The cells were scratched with a pipette tip and left for 60 minutes to migrate into the wound. Immunofluorescence was completed by staining with an antibody against SLK (green) and DAPI (blue). The scale bar represents 10 μ m and applies to all panels. N=3.

(Mansour, Ravdin, & Dressler, 1994). Cross-talk between the HER2 and FAK/c-src signaling pathways has also been consistently demonstrated (Benlimame, He, Jie, Xiao, Xu, & Loignon, 2005) (Dankort, Wang, Blackmore, Moran, & Muller, 1997) (Dankort, Jeyabalan, Jones, Dumont, & Muller, 2001a) (Dankort, et al., 2001b) (Roovers, et al., 2009). Supporting a role for SLK downstream of HER2, a kinase inactive SLK mutant (SLK^{K63R}) dramatically reduces Heregulin-induced cellular migration, which signals through the HER2 receptor (Roovers, et al., 2009). Our lab has also shown that SLK kinase activity is increased when stimulated with Heregulin (Roovers, et al., 2009) (Dankort & WJ, 2000) (Mansour, Ravdin, & Dressler, 1994) (Nanni, et al., 2000) (Schechter, Stern, Decker, Drebin, Greene, & Weinberg, 1984). Therefore, a link can be drawn between SLK and HER2 in cellular migration signaling pathways. A schematic of SLK and where it localizes in cellular migration and invasion is shown in **Figure 3**. First, SLK is activated and recruited to the focal adhesion complex where it interacts with paxillin. Next, paxillin is phosphorylated by SLK at serine 250 (Quizzi, et al., 2013). This key phosphorylation event leads to focal adhesion turnover and increased migration and invasion. The specific mechanisms of crosstalk between SLK, HER2 and focal adhesion pathways have yet to be fully understood.

It has recently been shown that SLK phosphorylates RhoA at serine 188, thereby inactivating RhoA and sequestering it in the cytosol, leading to increased vasodilation following stimulation of the angiotensin II type 2 receptor (Guilluy, et al., 2008). Although our proposed research does not fall within the same cell type, we believe that the phosphorylation of RhoA by SLK could potentially play a role in the non-canonical TGF β -mediated pathway of EMT. One potential mechanism involves SLK affecting polymerization of actin into stress fibers and breakdown of epithelial tight junctions. Cellular migration and invasion in breast cancers are

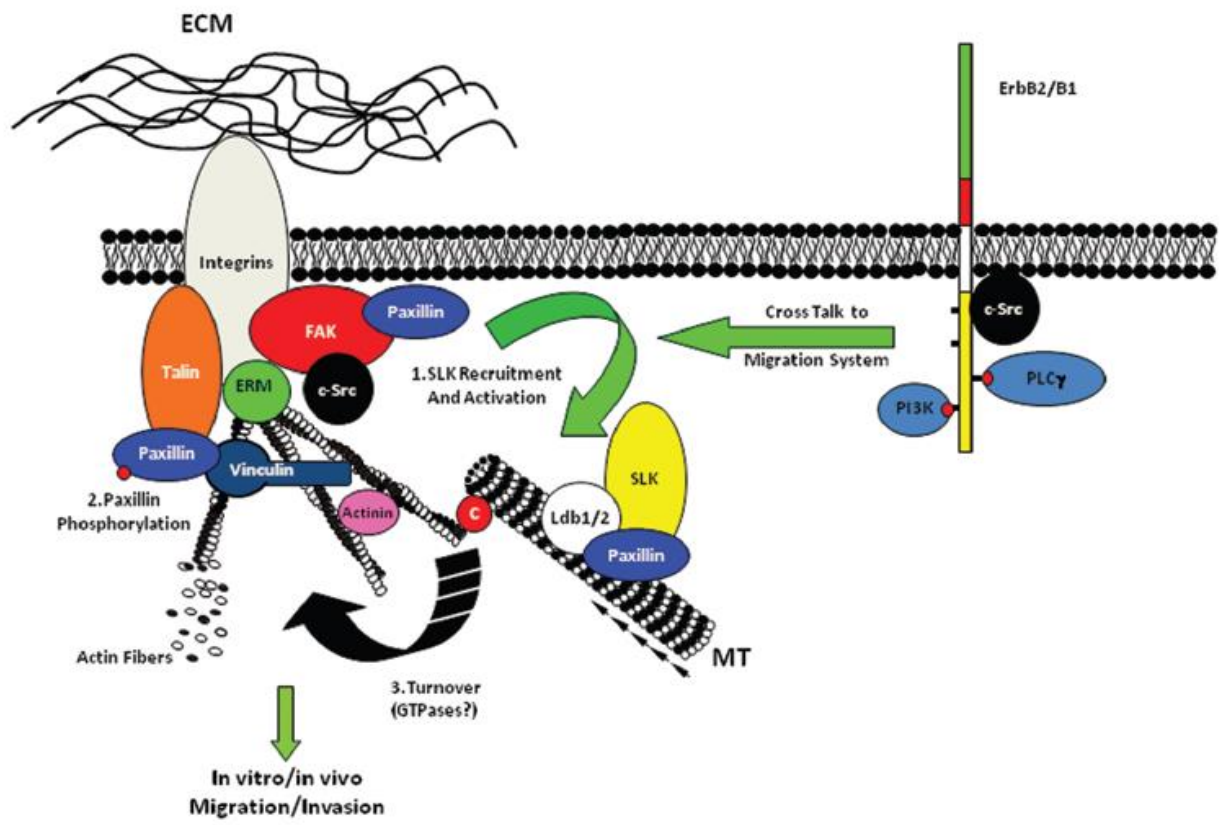


Figure 3. The localization of SLK in migration and invasion pathways involving crosstalk between Erb-B2 and focal adhesion turnover signaling. Initiation of cellular migration or adhesion begins with the integrin/FAK/c-src complex which recruits a large number of adapter proteins. SLK becomes activated through the microtubule network, thus allowing focal adhesion turnover to progress (Wagner, et al., 2008). It has also been demonstrated that SLK can phosphorylate paxillin which induces focal adhesion turnover (Quizil, et al., 2013). The Erb-B2 signaling pathway has also been shown to crosstalk with the migration and invasion signaling pathway by signaling through SLK and receptor-bound integrins (Roovers, et al., 2009). Figure adapted with permission from Al-Zahrani, Baron, & Sabourin, 2013.

increased downstream of TGF β signaling through a signaling pathway called epithelia-to-mesenchymal transition (Miettinen, Ebner, Lopez, & Derynck, 1994) (Mansour, Ravdin, & Dressler, 1994). There are many other TGF β pathways that lead to EMT signaling, and will be discussed in more detail as they relate to the role of SLK.

1.2 The Hallmarks of Cancer

1.2.1 Genetic Mutations

Cancer development has been characterized by the presence of six abnormal traits that contribute to a distinct phenotype and genotype. All forms of cancer are associated with evading apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, neoangiogenesis, self-renewal, and tissue invasion and metastasis. It involves an accumulation of mutations by means of uncontrolled cell division, leading to a defect in normal tissue function. It has been well characterized that three categories of genes regulate the cell cycle: oncogenes, group I tumor suppressor genes, and group II tumor suppressor genes. The first group, oncogenes, act as a positive growth signal by stimulating entry of cells into the cell cycle and out of quiescence. The dysregulation of this process due to mutations in these genes results in a phenotype that promotes cell division and replication. In a non-cancer context, group I tumor suppressor genes act as negative growth signals. Mutations in the second group result in uncontrolled cell division that can progress without any external stimuli. Finally, group II tumor suppressor genes are important for DNA repairs and apoptosis. In cancer, mutations in the genome accumulate over time due to the inactivation of these genes. This results in a propagation of the tumorigenic phenotype and prevents cells from inducing apoptosis. Therefore, mutations in all three sets of genes ultimately leads to uncontrolled and unregulated cell division that is

characteristic of cancer development (reviewed in Hanahan & Weinberg, 2000) (Hanahan & Weinberg, 2011).

1.2.2 Invasion and Metastasis

Metastasis is the cause of 90% of human cancer deaths, indicating the importance of research in this field to identify the underlying mechanisms of this process (Sporn, 1996) (reviewed in Hanahan & Weinberg, 2000). For cells to gain this metastatic capacity, they must first grow as a primary tumor localized to one tissue region. A primary tumor is defined as a group of cells that can be differentiated from the normal cells by their shape, size and genomic sequence (Hanahan & Weinberg, 2000). Tumors that remain localized to one region of the body are categorized as benign tumors. In contrast, invasive tumors can grow large enough to spread throughout the body and re-establish in distal sites by manipulating the use of lymphatic and circulatory systems (Hanahan & Weinberg, 2000). A key signaling pathway that enables the relocation of invasive tumors is referred to as epithelial-to-mesenchymal transition (EMT). This process involves cytoskeletal and genetic changes within a cell to encourage a more motile and invasive phenotype (Miettinen, Ebner, Lopez, & Derynck, 1994).

For cancer cells to successfully metastasize to a distant site in the body, they must follow an established set of steps called the invasion-metastasis cascade. Cells must first breach the basement membrane (BM), intravasate into the lumen of blood or lymph vessels, evade the body's immune cells in circulation, extravasate into a distant tissue, and re-colonize at a distant site (reviewed in Valastyan & Weinberg, 2011). The reverse process of EMT, mesenchymal-to-epithelial transition (MET), is also an important process required for regenerating tumor growths at a secondary site (reviewed in Hugo, et al., 2007).

The invasion of primary tumor cells begins with a breach in the basement membrane (BM), a key structure involved in regulating epithelial cell behaviour (reviewed in Kalluri,

2003). Two forms of invasion were characterized based on the different structural components. Mesenchymal invasion is associated with proteases, actin stress fibres and integrins, whereas amoeboid invasion is not (Lewis, 1934) (Entschladen, Niggemann, Zanker, & Friedl, 1997) (Friedl, Borgmann, & Brocker, 2001) (Friedl & Wolf, 2003). Direct invasion of the BM is facilitated by the production of MMPs (matrix metalloproteinases) that degrade the basement membrane and activate TGF β (transforming growth factor- β) through proteolytic cleavage (Yu & Stamenkovic, 2000).

1.3 Cellular Migration

Cellular migration is a critical process important in many pathways, including the inflammatory response, embryogenesis, and tissue repair and regeneration (Horwitz & Lauffenburger, 1996) (Webb, Parsons, & Horwitz, 2002). In cancer, tumors can hijack normal cellular migration signaling pathways, characterized by a more motile and invasive disease in patients (Wells, 2000) (Wang, Goswami, Sahai, Wyckoff, Segall, & Condeelis, 2005). Cell migration requires the activation of multiple protein complexes. This process begins with stable focal adhesions (FAs) being formed at the leading edge of extensions that reach outwards into the environment (Izzard & Lochner, 1980) (Zaidel-Bar, Ballestrem, Kam, & Geiger, 2003). Cell membranes extend active processes, termed lamellipodia and filopodia, by actin polymerization (Condeelis, 1993) (Stossel, 1993). The FAs are multiprotein complexes consisting of the phospho proteins FAK (focal adhesion kinase), paxillin and tensin that support the cell structure during migration (Lo, Weisberg, & Chen, 1994) (Schaller & Parsons, 1994) (Turner, 1994). FAK plays an important role in the assembly of adhesion complexes, and contains binding sites for paxillin, talin, and integrin, which are key effectors of cell migration (Schaller & Parsons, 1994) (Chen, Appeddu, Parsons, Hildebrand, Schaller, & Guan, 1995) (Chen & Guan, 1994). The

cellular migration machinery is continuously assembled at the leading edge, and disassembled at the cell rear by means of actin contraction, thus propelling the cell forward. This protrusive force and subsequent contractile force are coordinated movements that facilitate cell migration (Chen W. , 1981). Therefore, changes in the actin cytoskeleton and key signaling through focal adhesions complexes result in the translocation of cells in a specific direction (Mitra & Schlaepfer, 2006) (Webb, Parsons, & Horwitz, 2002) (As reviewed in Lauffenburger & Horwitz, 1996).

Focal adhesion turnover is a complex process that requires integrins, a family of transmembrane receptors important in many cellular processes (Zaidel-Bar, Ballestrem, Kam, & Geiger, 2003). Integrins are a key family of receptors that are essential for assembling focal adhesion complexes in cell migration. They activate two main protein kinases, focal adhesion kinase (FAK) and c-src proto-oncogene, by bridging the extracellular matrix and the intracellular actin cytoskeleton (Webb, Parsons, & Horwitz, 2002). It has been well documented that integrin/FAK/c-src complexes are crucial in the signaling processes underlying FA turnover (Mitra & Schlaepfer, 2006). For example, studies have shown that FAK and c-src null mice display reduced cell migration, spreading, and motility, suggesting an integrin-dependent activation of FAK and c-src during cellular migration (Chen, Tzen, Bresnick, & Chen, 2002).

Another critical aspect of cellular migration is the reorganization of the actin cytoskeleton by the Rho GTPases. During cell migration, RhoA (ras homolog gene family member A) has been shown to regulate critical processes in the mobilization of the actin cytoskeleton (Edlund, Landstrom, Heldin, & Aspenstrom, 2002). Within the signaling pathway, the small GTPase, RhoA, activates ROCK (Rho-associated-coiled-coil-containing protein kinase), leading to the formation of actin stress fibres (Bhowmick, et al., 2001) (Edlund, Landstrom, Heldin, &

Aspenstrom, 2002) (Haynes, Srivastava, Madson, Wittmann, & Barber, 2011). RhoA has also been shown to be important for disrupting E-cadherin expression in the adherens junctions downstream of TGF β signaling (Bhowmick, et al., 2001) (Tavares, Mercado-Pimentel, Runyan, & Kitten, 2006) (Cho & Yoo, 2007). Another key player implicated in cytoskeletal reorganization involves the p38MAPK (mitogen-activated protein kinase) pathway (Hannigan, Zhan, Ai, & Huang, 1998) (Huot, Houle, Rousseau, Deschesnes, Shah, & Landry, 1998) (Bhowmick, Zent, Ghiassi, McDonnell, & Moses, 2001), where activation of p38MAPK is regulated by Rac1, a small GTPase belonging to the RAS superfamily (Didsbury, Weber, Bokoch, Evans, & Snyderman, 1989) (Polakis, Weber, Nevins, Didsbury, Evans, & Snyderman, 1989). In TGF β -induced EMT, p38MAPK has been shown to induce actin polymerization through phosphorylation of heat shock protein (HSP)-27 (Hedges, et al., 1999) (Pichon, Bryckaert, & Berrou, 2004). There are many key pathways involved in the cytoskeletal changes that work in concert to promote cell migration.

In addition to RhoA and Rac1, Cdc42 has also been shown to play a critical role in filopodial extension and cell migration (Edlund, Landstrom, Heldin, & Aspenstrom, 2002). The Rho GTPases exert their actin remodelling activity through the direct binding of the GTP-bound GTPase to target kinases such as MRCK1, PAKs (p21-activated kinase), and ROCK (Edlund, Landstrom, Heldin, & Aspenstrom, 2002) (as reviewed in Sit & Manser, 2011). The Rho family of proteins are subsequently inactivated by the activation of their intrinsic GTPase activity stimulated by specific GAPs (GTPase-activating protein) (Edlund, Landstrom, Heldin, & Aspenstrom, 2002) (Cherfils & Zeghouf, 2012). The pathways, in concert with other actin remodelling activities such as Arp2/3 and WASP, work in concert to promote cell migration (as reviewed in (Cherfils & Zeghouf, 2012)).

1.4 HER2/Neu/ErbB2 Expression in Breast Cancer

The HER2/Neu/ErbB2 transmembrane receptor (human epidermal growth factor receptor), found in humans, rats, and mice respectively, is overexpressed in 30% of primary human breast cancers (Mansour, Ravdin, & Dressler, 1994). Studies show that mice overexpressing ErbB2 are diagnosed with a more invasive and metastatic disease (Hynes & Lane, 2005). The equivalent oncoprotein in humans, HER2, belongs to the epidermal growth factor receptor family of proteins. Signaling downstream of HER2 requires its dimerization with other key transmembrane receptors including EGFR, HER3, or HER4 (Siegel, Shu, Cardiff, Muller, & Massagué, 1999). Activation of HER2 kinase activity induces the phosphorylation of the cytoplasmic tail of HER2 in five distinct locations: Y1144, Y1201, Y1226/7, and Y1253 (Dankort, Wang, Blackmore, Moran, & Muller, 1997). This phosphorylation event results in the initiation of cell migration and survival pathways.

Research has demonstrated links between the HER2 and EMT signaling pathways. EMT is a normal cellular process that can be stimulated using the cytokine TGF β . Highly mutated cancer cells hijack this signaling pathway, promoting increased migration and invasion (Miettinen, Ebner, Lopez, & Derynck, 1994). Inhibition of the HER2 receptor with a neutralizing antibody leads to a decrease in migration following TGF β treatment, suggesting that HER2 signaling is required for EMT (Ueda, Wang, Dumont, Yi, Koh, & Arteaga, 2004). Additionally, activation of the EMT pathway has been linked to the overexpression of the HER2 oncoprotein (Khoury, Dankort, Sadekova, Naujokas, Muller, & Park, 2001). HER2 signaling also results in an increase in TGF β secretion, thus propagating EMT and resulting in more motile cells (Wang S. , 2011). This promotes cancer progression through activation of the FAK/c-src signaling pathway associated with cell migration (Wang, Xiang, Zent, Quaranta, Pozzi, & Arteaga, 2009).

In addition, FAK signaling is required downstream of HER2 activation, which is necessary for initiating cell migration (Benlimame, He, Jie, Xiao, Xu, & Loignon, 2005). Therefore, it has been well documented that HER2, TGF β and the focal adhesion signaling pathways crosstalk and promote increased migration and invasion in cancer.

1.5 Epithelial-to-mesenchymal transition

1.5.1 The Epithelial Phenotype

Epithelial-to-mesenchymal transition, hereafter referred to as EMT, is essential for normal growth processes including wound healing and tissue regeneration (Kalluri & Weinberg, 2009) (Thiery, Acloque, & Huang, 2009). EMT plays an important role in embryogenesis and tissue morphogenesis of key tissues such as the heart and the peripheral nervous system (Huber, Kraut, & Beug, 2005).

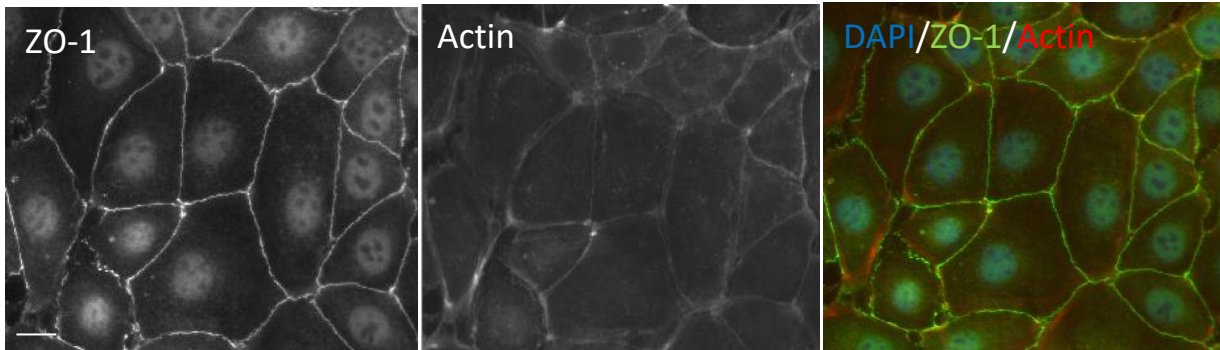
The structure of epithelial cells involves interactions between several specialized structures: desmosomes, adherens junctions, gap junctions, and tight junctions (Balda & Matter, 1998). Tight junctions (TJs) are important for maintaining epithelial cell structure, and link them together with occludin and claudin proteins. These complexes are located between the apical and basolateral membranes of cells, and are responsible for organizing a semi-permeable membrane at the cell junctions (Aijaz, Balda, & Matter, 2006). In addition, TJs link the ZO proteins to the actin cytoskeleton to provide structural support to the cells (Tsukita, Furuse, & Itoh, 2001). The second group of proteins that contribute to the epithelial phenotype are the adherens junctions that contain E-cadherin as a key component (Niessen & Gottardi, 2008) (Wheelock & Johnson, 2003) (Hanahan & Weinberg, 2000). Cadherins are calcium-dependent transmembrane glycoproteins that interact with α , β -catenin and p120 to form secure connections to the actin cytoskeleton (Christofori, 2003) (Beavon, 2000) (Aijaz, Balda, & Matter, 2006).

They also function to prevent initiating metastatic and invasion signaling pathways of epithelial cells (Berx, et al., 1995) (Hanahan & Weinberg, 2011). However, an accumulation of mutations in cancer progression can alter E-cadherin expression and transform epithelial cells into an invasive and motile phenotype (Imamichi & Menke, 2007). Desmosomes consist mainly of desmocollin and desmoglein, and are linked to the intermediate filaments of the cytoskeleton by plakoglobin, plakophilin, and desmoplakin (Garrod & Chidgey, 2008). Finally, gap junctions are a specialized structure made up of connexin proteins that allow the passage of ions between adjoining epithelial cells (Evans & Martin, 2002). Together, tight junctions, adherens junctions, desmosomes and gap junctions all contribute to the structural integrity of epithelial cells by maintaining cell quiescence and stabilizing the actin skeleton (Aijaz, Balda, & Matter, 2006).

1.5.2 The Mesenchymal Phenotype

EMT is associated with the development of secondary tumors and cancer metastasis (Thiery, 2002). Invasive tumors are capable of hi-jacking the body's lymphatics and circulatory systems to re-establish tumor growth in distant sites (Hugo, et al., 2007) (Moustakas & Heldin, 2007). To become mesenchymal, epithelial cells experience three distinct changes in cell morphology: a decreased expression of epithelial markers, an increased expression of mesenchymal markers and a re-organization of cytoskeletal actin into stress fibers (Miettinen, Ebner, Lopez, & Derynck, 1994). The disappearance of E-cadherin is considered a hallmark of EMT and indicates the transition to a mesenchymal phenotype (Thiery, 2002) but it is not the sole event required for cancer invasion and metastasis (Llorens, et al., 1998). The loss of E-cadherin expression was first observed as a result of EMT at the gastrulation stage in a chick (Edelman, Gallin, Delouvee, Cunningham, & Thiery, 1983). **Figure 4** demonstrates the key differences between epithelial and mesenchymal cells.

Untreated NMuMG epithelial cells



NMuMG epithelial cells +TGF β (48 hours)

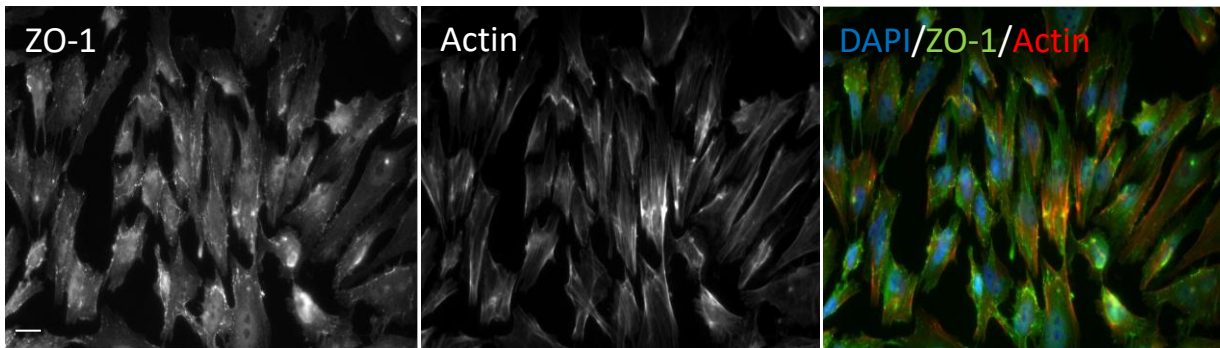


Figure 4. Notable phenotypic differences between epithelial and mesenchymal cells. Epithelial cells maintain characteristic cell-cell contacts using tight junctions, adherens junctions and desmosomes. **(A)** Immunofluorescence staining of NMuMG cells was done using antibodies against ZO-1 (green) and actin (red). **(B)** NMuMG cells were treated with 2ng/mL of TGF β for 48 hours to stimulate EMT. Immunofluorescence staining was done using antibodies against ZO-1 (green) and actin (red). Scale bar represents 10 μ m and applies to all panels.

There are three types of EMT that control different processes in the body. Type I is an essential, normal developmental process that occurs during embryogenesis. More specifically, it facilitates formation of the mesoderm in the developing embryo (Shook & Keller, 2003). Type II is involved in wound healing and tissue regeneration (Lopez-Novoa & Nieto, 2009). The third type occurs when a tumor hijacks and modifies a normal signaling pathway following the accumulation of mutations (Thiery, Acloque, & Huang, 2009) (Principe, et al., 2014). The EMT signaling pathway that is responsible for tumor migration can be induced by a secreted cytokine transforming growth factor beta-1 (TGF β -1) both *in vivo* and *in vitro* (Miettinen, Ebner, Lopez, & Derynck, 1994). EMT can also be stimulated by many other factors including EGF (epidermal growth factor), HGF (hepatocyte growth factor), FGF (fibroblast growth factor), BMPs (bone morphogenetic proteins), Notch, Wnt, TNF- α (tumor necrosis factor- α), and multiple other cytokines (Gavert & Ben-Ze'ev, 2008) (Piedra & Ros, 2002).

1.6 Transforming growth factor- β

1.6.1 TGF β Superfamily

The transforming growth factor- β (TGF β) superfamily consists of growth factor polypeptides that are involved in critical signaling processes within cells. This superfamily is grouped into multiple categories that include the TGF β 's, activins and inhibins, bone morphogenetic proteins (BMPs), glial cell-line derived neurotropic factors, (GDNFs), Mullerian inhibitory substance (MIS), and growth and differentiation factors (GDFs) (Kingsley, 1994). The activins and inhibins were first characterized based on their ability to regulate endocrine functions by increasing pituitary hormone secretion (Ling, et al., 1986), and are known to be secreted by the gonads (Lee, Mason, Schwall, Szonyi, & Mather, 1989). The BMP family was named as such because it is directly involved in *de novo* bone formation in muscle tissue

(Wozney, et al., 1988). The TGF β family was first characterized based on their ability to transform normal kidney fibroblasts into motile cells *in vitro* (Associati, Grotendorst, Miller, & Sporn, 1984) (Roberts & Sporn, 1985). It was further characterized as an essential protein in embryogenesis, inflammation, repair, and carcinogenesis (Roberts, et al., 1990) (Roberts & Sporn, 1987) (as reviewed in Mehra & Wrana, 2002).

1.6.2 Receptors

The TGF β superfamily has been shown to bind to three groups of receptors (Type I, Type II, and Type III), first identified using radioactive-labelled TGF β that made cross linkages to cell-surface proteins (Massague & Like, 1985). The TGF β receptors are transmembrane glycoprotein serine-threonine receptor kinases with key extracellular and intracellular signaling domains. Type II receptors are capable of autophosphorylation, and are also responsible for phosphorylating type I receptors during signaling (Wrana, Attisano, Wieser, Ventura, & Massague, 1994). The receptors specific to TGF β 1 include the TGF β Receptor Type I (T β RI), first identified as activin receptor-like kinase 5 (ALK-5) (Franzen, et al., 1993) (Yamashita, ten Dijke, Franzen, Miyazono, & Heldin, 1994b), and TGF β receptor II (T β RII) (Lin, Wang, Ng-Eaton, Weinberg, & Lodish, 1992) (as reviewed in (Mehra & Wrana, 2002)).

1.6.3 Function of TGF β in cancer

TGF- β can act as both a tumor suppressor and a tumor promoter. In early cancer development, it suppresses tumor proliferation by preventing entry into the G1 phase of the cell cycle. By recruiting cyclin-dependent kinase (CDK) inhibitors and suppressing c-Myc, a key component in cell growth and division, TGF β inhibits cell cycle progression (Massagué, 2008). Other studies have shown that TGF β signaling promotes apoptosis in cells by inducing the

expression of death-associated protein kinase (Jang, Chen, Chen, Chen, Su, & Chen, 2002) (Shi & Massagué, 2003). Therefore, TGF β acts as a tumor suppressor in normal cells.

However, an accumulation of mutations in cancer cells leads to changes in the TGF β signaling pathway. Many cancers present with mutations on the TGF β receptors and their downstream effectors, thus disrupting the protective tumor suppressor mechanisms (Gold & Parekh, 1999). This can result in a functional switch that ultimately drives tumor progression. In many cancers, tumor cells secrete TGF β and participate in autocrine and paracrine signaling in a positive-feedback loop (Derynck, et al., 1987) (Dickson, et al., 1987) (Bierie & Moses, 2006). In addition, growing tumors attract components of the body's immune system, including leukocytes and macrophages. An increase in these inflammatory response elements leads to an increase in TGF β secretion, therefore creating an accumulation at the tumor site and an increase in TGF β pro-migratory signaling (Massagué, 2008). Therefore, it is due to the loss of the tumor-suppressive action of TGF- β , and the gain of a tumor-promoter function that enables cancer progression and promotes the transformation into a motile phenotype. In summary, overexpression of TGF- β due to an accumulation of mutations in cancer cells contributes to tumor progression (Bierie & Moses, 2006) (Massagué, 2008).

1.7 TGF β Signaling Pathways

1.7.1 Smad-dependent pathway

EMT can induce morphological changes through two signalling pathways: the Smad-dependent (canonical) pathway, and the Smad-independent (non-canonical) pathway. The Smad-dependent pathway involves intracellular substrates known as Smads, first identified in *Drosophila* and *C.elegans* as the *dpp* gene (Padgett, St Johnston, & Gelbart, 1987). The first molecule isolated from the DPP (decapentaplegic) pathway was *Mad* (*Mothers-against-*

decapentaplegic) (Sekelsky, Newfeld, Raftery, Chartoff, & Gelbart, 1995). Homologs of the *Mad* genes were isolated in *C. elegans* and referred to as *sma-2, 3 and 4*, which were found downstream of a TGF β -like receptor (Savage, et al., 1996). In recognition of the origins of these proteins, the vertebrate homologs were classified as Smads after the *sma* and *Mad*-related genes (Derynck, et al., 1996). There are three groups of Smads characterized by their functions: the R-Smads (receptor-regulated Smads), the C-Smads (common Smads) and the I-Smads (inhibitory Smads) (Derynck, et al., 1996).

Signaling through the Smad-dependent pathway is initiated by the TGF β ligand, which binds to TGF β receptor II (T β RII), a constitutively active serine-threonine transmembrane kinase (Wrana, Attisano, Wieser, Ventura, & Massague, 1994). This results in the recruitment of a second serine-threonine kinase TGF β receptor I (T β RI) and its subsequent activation through transphosphorylation by T β RII. The T β R's both exist as homodimers, and associate together as two T β RII's and two T β RI's (Huang, et al., 2011). The receptor complex is then internalized by means of a clathrin-coated pit or a lipid raft-caveolae depending on the cellular context. In the former pathway, the complex is brought to an early endosome where the SMAD anchor for receptor activation (SARA) and cytoplasmic form of promyelocytic leukemia protein (cPML) assist in recruiting the downstream effectors of this pathway to propagate the signal (Neuzillet, et al., 2015) (Papageorgis, 2015). The activated receptor complex then leads to activation of the R-Smads (Smad 2 and Smad 3) through direct phosphorylation by T β RI (Eppert, et al., 1996) (Nakao, Roijer, Imamura, Souchelnytskyi, Stenman, & Heldin, 1997b) (Yingling, Das, Savage, Zhang, Padgett, & Wang, 1996). Following this activation, Smad2/3 forms a trimer complex with Smad 4 (Common-Smad) and translocates into the nucleus where they bind to DNA modulate gene expression (Baker & Harland, 1996) (Hoodless, et al., 1996). Specifically, Smad4

binds DNA following stimulation by TGF β (Yingling, Datto, Wong, Frederick, Liberati, & Wang, 1997). This complex recruits transcriptional co-repressors that block transcription of epithelial genes including E-cadherin, claudins, occludin, desmoplakin and plakoglobin. The trimer Smad2/3/4 also recruits transcriptional co-activators to upregulate mesenchymal markers such as Snail1, Snail2, fibronectin, vimentin, N-cadherin and MMPs (matrix metalloproteinases) (Xu, Lamouille, & Derynck, 2009). The result of this signaling pathway contributes to the phenotypic changes observed in the EMT process.

The inhibitory Smads, Smad 6 and Smad7, work in a negative feedback loop to inhibit Smad activation. In contrast to the R-Smads, Smad7 translocates from the nucleus to the cytoplasm to exert its effects on the receptor (Itoh, et al., 1998). Smad7 is used as an adapter and facilitates the binding of both Smurf1 (Smad ubiquitination regulatory factor 1) and Smurf2 to the receptor complex, resulting in their degradation and halting the signaling (Ebisawa, et al., 2001) (Kavsak, et al., 2000). In addition, Smads 6 and 7 become activated and prevent further signaling through this pathway by binding directly to T β RI, therefore blocking Smads and preventing their activation (Hayashi, et al., 1997) (Imamura, et al., 1997) (Nakao, et al., 1997). The inhibitory Smads are also responsible for Smads2/3/4 protein degradation by covalently linking the target protein to a ubiquitin protein that is then recognized by the proteasome and destroyed. The ligation of the ubiquitin to the target protein is accomplished through E3 ubiquitin ligases, Smurf 1 and Smurf 2 (Zhang, Chang, Gehling, Hemmati-Brivanlou, & Derynck, 2001) (Zhu, Kavsak, Abdollah, Wrana, & Thomsen, 1999). A schematic of the Smad-dependent pathways is shown in **Figure 5**.

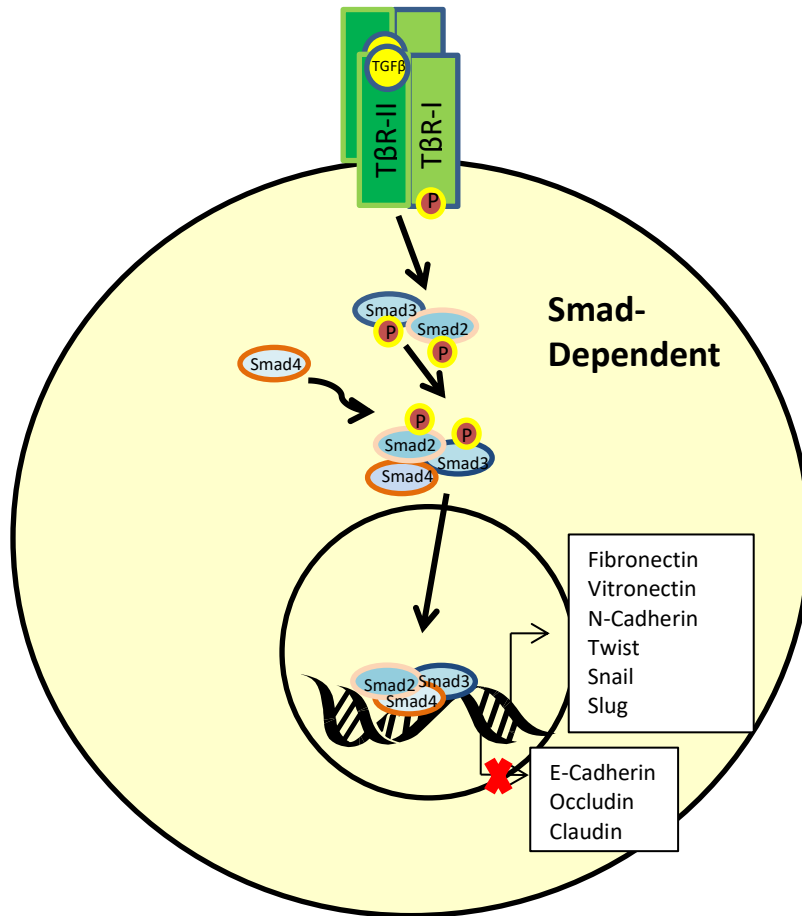


Figure 5. The Smad-dependent pathway in transforming growth factor- β -dependent epithelial-to-mesenchymal transition. To initiate signaling, the TGF β ligand binds to TGF β RII which then associates with and subsequently phosphorylates TGF β RI. The receptor Smads, Smad2 and Smad3, are directly phosphorylated by TGF β RI and form a complex with the co-Smad, Smad4. The Smads then translocate into the nucleus where they associate with other co-transcription factors to upregulate mesenchymal markers and downregulate epithelial markers. Negative feedback from this pathway involves the inhibitory Smad, Smad 7, which recruits E3 ubiquitin ligases to initiate degradation of the receptor complex, leading to its depletion (not shown).

1.7.2 Gene expression during EMT

There are three key families of transcription factors that are activated downstream of TGF β signaling: Snail, Twist, and ZEB families (Peinado, Olmeda, & Cano, 2007). The Snail family includes Snail1, Snail2 (Slug), and Snail3 (Smuc) transcription factors which consist of a distinct C-terminal domain with 4-6 zinc fingers, required for binding E-box elements to regulate gene transcription, and an N-terminal SNAG (Snail/Gfi) domain which associates with co-repressors (Batlle, et al., 2000) (Peinado, Ballestar, Esteller, & Cano, 2004). The transcriptional regulation of Snail1 is mediated by multiple pathways that work in concert to upregulate its expression. Studies demonstrate that there are multiple transcription factors that bind to the Snail1 promoter including the Smad proteins, NF- κ B, HMGA2, PARP-1, STAT3, and many others (Kaufhold & Bonavida, 2014). Downstream of TGF β 1 treatment, the Smad proteins (Cheng, Chang, & Leung, 2013) (Horiguchi, Shirakihara, Nakano, Imamura, Miyazono, & Saitoh, 2009), IKK α (Brandl, et al., 2010) (Julien, et al., 2007) and HMGA2 (Thuault, Tan, Peinado, Cano, Heldin, & Moustakas, 2008) have been shown to bind directly to the Snail1 promoter. Snail1 has also been shown to bind to its own promoter (Peiro, et al., 2006). Once upregulated, Snail1 is subject to post-translational modifications that affect its localization and stability. In the nucleus, Snail1 is responsible for mediating the expression of multiple mesenchymal gene targets, including vimentin, fibronectin, and MMP2/9 (matrix metalloproteinase) (reviewed in Cano, et al., 2000). Snail is also responsible for silencing other epithelial gene markers including claudins 1,3,4 and 7, and Snail1 and Snail2 are both associated with repression of occludin (De Craene, Gilbert, Stove, Bruyneel, van Roy, & Berx, 2005) (Ikenouchi, Matsuda, Furuse, & Tsukita, 2003) (Kajita, McClinic, & Wade, 2004) (Wang, et al., 2007). It has also been well characterized that both Snail1 and Snail 2 bind directly to the E-box

element of the E-cadherin gene *CDH1* resulting in its inactivation (Batlle, et al., 2000) (Cano, et al., 2000) (Herranz, et al., 2008) (Hemavathy, Guru, Harris, Chen, & Ip, 2000) (Bolos, Peinado, Perez-Moreno, Fraga, Esteller, & Cano, 2003) (Hajra, Chen, & Fearon, 2002). Finally, Snail1 has been shown to disrupt the Crumbs3 complex, an important structure involved in maintaining epithelial cell polarity, by suppressing its transcription (Whiteman, Liu, Fearon, & Margolis, 2008).

The second major group of transcription factors associated with TGF β -induced EMT is the ZEB (zinc finger E-box-binding homeobox) family, which include two members in vertebrates: ZEB1 and ZEB2. ZEB1, in particular, interacts with the receptor Smads to enhance TGF β signaling (Postigo, 2003). Their structure consists of two zinc finger domains located on both ends of the protein, which allows them to bind to the E-box element of a target gene. The N-terminal domain consists of 4 zinc fingers, and the C-terminal domain consists of 3 zinc-fingers (Verschueren, et al., 1999). ZEB1 can repress gene transcription by associating with the C-terminal binding protein (CTBP). It can also lead to gene activation by associating with different transcription factors, the p300/CBP associated factor (PCAF) and p300 (Postigo, Depp, Taylor, & Kroll, 2003) (Peinado, Olmeda, & Cano, 2007). The ZEB proteins have also been shown to repress E-cadherin expression independently of the Snail transcription factors (Shirakihara, Saitoh, & Miyazono, 2007) (Eger, et al., 2005). Additionally, ZEB2 decreases Claudin-4 and ZO-3 expression (Vandewalle, et al., 2005), and increases the mesenchymal markers vimentin (Bindels, et al., 2006), N-Cadherin (Vandewalle, et al., 2005), and MMP2 (Taki, Verschueren, Yokoyama, Nagayama, & Kamata, 2006). Upregulation of both ZEB1 and ZEB2 leads to an increase in the migratory and invasive capacity of cells (Comijn, et al., 2001) (Vandewalle, et al., 2005) (Spaderna, et al., 2008).

The third major group of transcription factors is the basic helix-loop-helix (bHLH) family that consists of seven categories, three of which are important for TGF β -associated EMT. Class I includes the E12 and E24 transcription factors which are important for E-cadherin repression through direct binding of the E-box, increased expression of vimentin and fibronectin, and increased migration and invasion (Bolos, Peinado, Perez-Moreno, Fraga, Esteller, & Cano, 2003) (Perez-Moreno, et al., 2001) (Kondo, et al., 2004). Class II, the Twists (1 and 2), have been shown to have increased expression in human tumors (Yang, Mani, & Weinberg, 2006), and are involved in decreasing E-cadherin expression, increasing vimentin and N-cadherin expression, and increasing migration and invasion (Yang, et al., 2004) (AnisEAU, et al., 2008). Class V, the Ids (inhibitor of DNA binding), suppress the actions of other transcription factors by associating with the Class I family, E12/E47 (Peinado, Olmeda, & Cano, 2007) (Massari & Murre, 2000).

Finally, HMGA2 (high mobility group A2) can also act to induce EMT by binding to AT-rich DNA in a Smad3/4 –dependent mechanism to induce Snail2 and Twist1 (Thuault, Valcourt, Petersen, Manfioletti, Heldin, & Moustakas, 2006) (as reviewed in (Xu, Lamouille, & Derynck, 2009).

1.7.3 Smad-independent pathways

EMT is also regulated by many other signaling cascades apart from the Smad-dependent pathway. Some of these pathways include signalling through Erk/JNK/p38MAPK, Rho GTPases, PI3K/Akt, Wnt and Notch pathways (Jordan, Landau, & Iyengar, 2000). The crosstalk that exists between the canonical and non-canonical signaling pathways is very complex and has yet to be fully understood.

The Ras/p38/JNK MAPK (mitogen-activated protein kinase) pathway is activated downstream of the TGF β receptors, and has been shown to work in conjunction with the Smad

signaling pathway to regulate gene expression (Funaba, Zimmerman, & Mathews, 2002) (Lehmann, et al., 2000) (Davies, Robinson, Smith, Huntley, Prime, & Paterson, 2005). Contrasting research has demonstrated that Erk (extracellular signal-related kinase) can phosphorylate the receptor Smad proteins (Smad 2 and 3) in their linker region, which prevents nuclear translocation (Kretzschmar, Doody, Timokhina, & Massague, 1999). Other studies have outlined a signaling pathway where ShcA (src homology 2 domain containing) is directly phosphorylated T β RI, recruiting Grb2 (growth factor receptor-bound protein 2) and Sos (son of sevenless) to activate the Ras, MEK1/2 and Erk proteins. The activation of MEK1/2 and Erk kinases upregulates the mesenchymal markers N-cadherin and matrix metalloproteinases, and downregulates the epithelial marker E-cadherin when co-stimulated with epidermal growth factor (EGF) (Grande, Franzen, Karlsson, Ericson, Heldin, & Nilsson, 2002) (Uttamsingh, et al., 2008). In addition, p38MAPK is phosphorylated downstream of the TGF β treatment, and has been associated with TGF β -induced EMT in isolation of the Smad proteins (Bakin, Rinehart, & Tomlinson, 2002) (Yu, Hebert, & Zhang, 2002). Finally, it has been demonstrated that JNK (Jun amino-terminal kinase) is linked to TGF β -induced EMT by associating with the Smads and inducing the expression of fibronectin, vimentin, and α -smooth muscle actin, as well as downregulating E-cadherin, and reorganizing cytoskeletal actin (Santibanez, 2006) (Liu, et al., 2008)

Another pathway associated with TGF β signaling involves the Rho GTPase, RhoA, and its effector ROCK (Rho-associated protein kinase). Research has shown that RhoA is activated downstream of TGF β , thereby activating ROCK, which induces polymerization of actin into stress fibres (Ishizaki, et al., 1996) (Leung, Manser, Tan, & Lim, 1995) (Leung, Chen, Manser, & Lim, 1996) (Pellegrini & Mellor, 2007). Independently of Smad activation, downregulating

RhoA expression prevents actin reorganization into stress fibres, and does not induce α -smooth muscle actin upon TGF β stimulation (Tavares, Mercado-Pimentel, Runyan, & Kitten, 2006) (Cho & Yoo, 2007) (Bhowmick, et al., 2001) . It has also been shown that Par6, a key regulator of cell polarity, gets phosphorylated by T β RII, allowing Smurf to degrade RhoA. This event leads to tight junction degradation downstream of TGF β signaling, and occurs independently of the Smad proteins (Ozdamar, Bose, Barrios-Rodiles, Wang, Zhang, & Wrana, 2005).

An additional signaling pathway that is associated with TGF β -induced EMT is the PI3K/Akt system, which acts independently of Smad activation (Bakin, Tomlinson, Bhowmick, Moses, & Arteaga, 2000) (Lee, Kwon, & Joo, 2004) (Rodriguez-Barbero, Dorado, Velasco, Pandiella, Banas, & Lopez-Novoa, 2006) (Lien, Usami, Chien, & Chiu, 2006) (Lin, et al., 2007) (Yeh, et al., 2008). It has been shown that PI3K binds directly to the TGF β receptors, thereby increasing its activity and triggering a signaling cascade that affects α -smooth muscle expression and E-cadherin downregulation (Yi, Shin, & Arteaga, 2005) (Bakin, Tomlinson, Bhowmick, Moses, & Arteaga, 2000) (Kattla, Carew, Heljic, Godson, & Brazil, 2008). Downstream of PI3K activation, Akt is phosphorylated, and subsequently activates mTOR (mechanistic target of rapamycin) and S6 kinase 1 which can then upregulate the Snail1 transcription factor (Sarbasov, Ali, & Sabatini, 2005) (Pon, Zhou, Cheung, Ngan, & Wong, 2008).

In addition to the pathways described above, there are also key signaling complexes involved in crosstalk downstream of the TGF β receptors. The Wnt pathway involves β -catenin, a key protein found in adherens junctions that link cadherins to the cytoskeleton (McCrea, Turck, & Gumbiner, 1991). Another key player is GSK3- β (glycogen synthase kinase 3- β), which has been shown to regulate β -catenin stabilization (He, Saint-Jeannet, Woodgett, Varmus, & David, 1995) (Pierce & Kimelman, 1995) (Dominguez, Itoh, & Sokol, 1995) (Peifer, Pai, & Casey,

1994) (Yost, Torres, Miller, Huang, Kimelman, & Moon, 1996). In the absence of Wnt signaling, GSK3- β phosphorylates β -catenin, targeting it for degradation by ubiquitination (MacDonald, Tamai, & He, 2009) (Clevers, 2006). Wnt ligand activates this pathway, leading to β -catenin accumulation and nuclear translocation, complexing with TCF/LEF1 factors and activating transcription (Nelson & Nusse, 2004) (Nawshad, Medici, Liu, & Hay, 2007) (Liebner, et al., 2004). This pathway works in conjunction with the canonical pathway, as it has been shown that Smad 2, Smad4 and LEF1 are required to upregulate vimentin and fibronectin expression (Nawshad, Medici, Liu, & Hay, 2007).

1.8 Purpose of Research

1.8.1 Rationale

Cell migration requires the activation of key components of the focal adhesion (FA) complex, leading to their turnover and cell motility (Horwitz & Parsons, 1999). SLK is activated downstream of the FAK/c-src signaling complex, and plays a key role in FA turnover and cell migration (Wagner, et al., 2008). SLK-mediated phosphorylation of paxillin on serine 250 is critical for focal adhesion turnover and motility (Quizzi, et al., 2013). In addition, SLK has been shown to activate downstream of HER2 signaling, an important pathway linked to cancer progression (Roovers, et al., 2009). EMT, a key signaling pathway in cancer metastasis, can be stimulated *in vivo* by TGF β (Miettinen, et al., 1994). Interestingly, TGF β has been linked to HER2 signaling through the FAK/c-src pathways and synergizes with HER2 to promote metastatic progression of breast tumors (Wang, et al., 2009). Together, these observations raise the possibility that SLK is required for TGF β -mediated EMT and the downstream cytoskeletal remodelling necessary to confer the invasive mesenchymal phenotype.

To analyze the role of SLK in EMT, we used a normal murine mammary gland cell line, NMuMG. This cell line was chosen because it is the best studied *in vitro* model due to its strong response following TGF β treatment, leading to a robust induction of EMT. NMuMG cells are estrogen and progesterone receptor negative with negligible levels of HER2 (Miettinen, Ebner, Lopez, & Derynck, 1994). Therefore, this is not a suitable cell line to study the link between SLK and HER2. Further research should investigate this crosstalk in a HER2⁺ model, such as SKBR3 or 4T1 cells.

1.8.2 Hypothesis

As SLK is required downstream of HER2 and the FAK/c-src complex for cell migration and invasion, we have investigated the role of SLK in TGF β -mediated EMT. The cross-talk between HER2-FAK-TGF β and the requirement for SLK in cell migration and invasion have led to the hypothesis that **SLK is required for transforming growth factor- β -induced epithelial-to-mesenchymal transition**. We propose that SLK does not play a role in the canonical signaling pathway, but affects another pathway that crosstalks downstream of TGF β signaling. **Figure 6** describes a potential model that demonstrates where we believe SLK might fit in non-canonical EMT signaling.

1.8.3 Objectives

To assess the role of SLK in EMT we have:

1. Investigated the expression and localization of SLK in mammary epithelial cells responsive to TGF β , and assessed the response of SLK to TGF β stimulation,
2. Tested the requirement for SLK in the genetic and cytoskeletal response to TGF β and
3. Partially characterized the activity-independent role of SLK in this response.

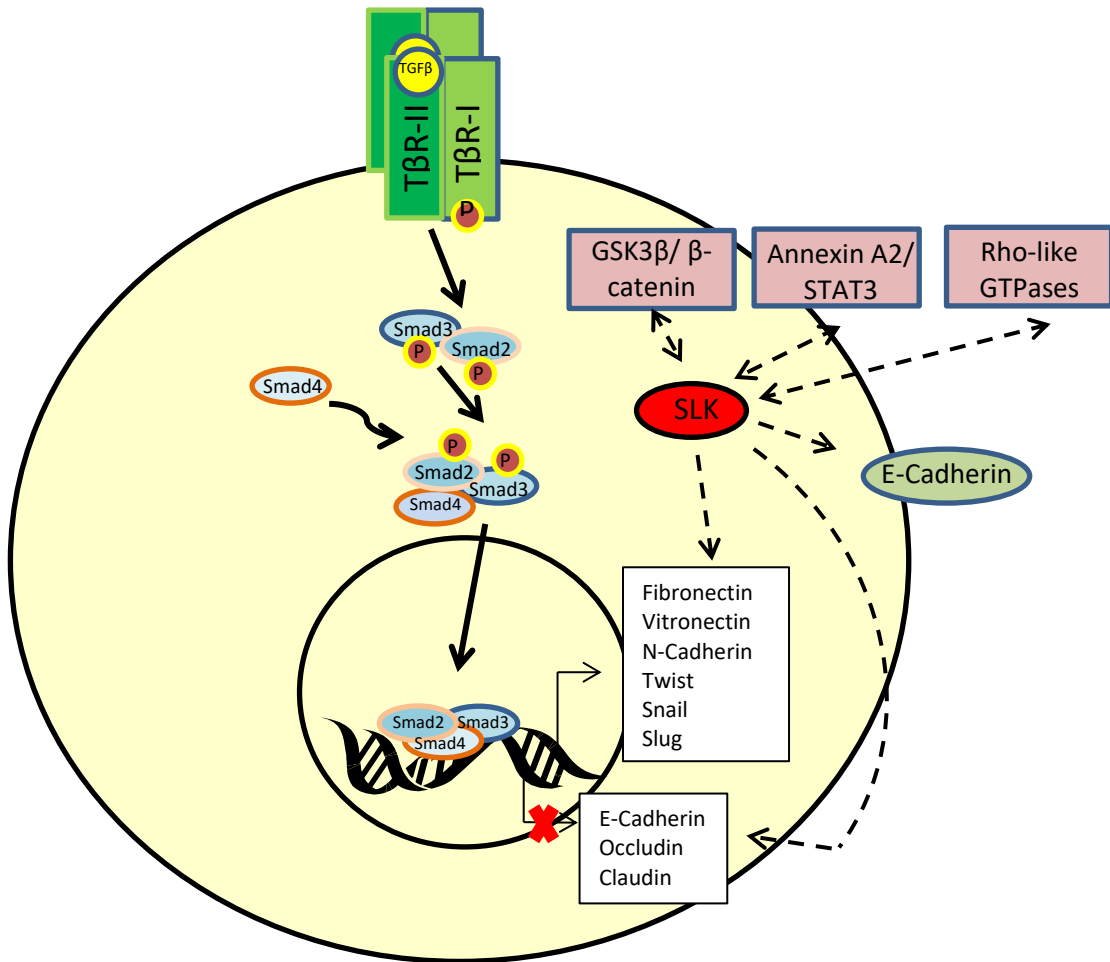


Figure 6. A potential model for SLK in non-canonical TGF β -dependent EMT. Our results demonstrate that SLK does not affect Smad3 phosphorylation or nuclear translocation. Therefore, we believe that SLK affects a non-canonical signaling pathway that leads to the observed changes in migration, invasion, and phenotype. Potential pathways that show promise include the GSK3 β / β -catenin, Annexin A2/ STAT3, or the Rho-like GTPases pathways.

2.0 MATERIALS AND METHODS

2.1 Cell Culture

NMuMG cells (ATCC, Manassas, Virginia, United States) were maintained in DMEM 1X (Dulbecco's Modification of Eagle's Medium), supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 10 μ g/mL insulin (Roche), 2mM L-glutamine (Invitrogen), and 200 U mL⁻¹ penicillin/streptomycin (Invitrogen). All experiments were performed in medium as described above but without the added insulin. The cells were grown in a humidified incubator set at 37°C and 5% CO₂. NMuMG cells were always serum-starved overnight prior to treatment with TGF β 1. TGF β 1 (Sigma-Aldrich) was added to cells at a concentration of 2ng/mL in serum-free media (1X DMEM, Corning, USA) in all experiments.

2.2 SLK Adenovirus Infection

To effectively knockdown SLK expression in NMuMG cells, a short hairpin adenovirus against SLK (AdshSLK), and a corresponding scramble control with a GFP tag were used at a concentration of 4.2 x 10¹⁰ pfu/mL. The targeting sequence for the shSLK adenovirus is 5'-GGTTGAGATTGACATATTA-3'. For infections, the cells were plated on a 10cm dish at 7.5 x 10⁵ cells/plate the day before adenovirus infection. On the day of the infection, the cells were first washed with 1X PBS (HyClone), and then re-suspended in serum-free DMEM to keep the cells in suspension. The sh (short hairpin) SLK adenovirus, and the corresponding scramble control virus was added to the suspended cells in serum-free media, and incubated at 37°C and 5% CO₂. The plates were gently agitated every 15 minutes for 90 minutes. Finally, the cells were re-fed with 10% FBS DMEM media (supplemented with 10% fetal bovine serum (Invitrogen), 2mM L-glutamine (Invitrogen), and 200 U mL⁻¹ penicillin/streptomycin (Invitrogen) and left to grow for a minimum of 48 hours before harvesting.

2.3 Protein Extraction and Western Blotting

For protein extraction, cells were washed in 5 mL of 1X PBS (diluted from 10X PBS stock: 0.72g KH_2PO_4 , 45g NaCl, 2.1g Na_2HPO_4 , and 500mL water), collected using a cell scraper into 0.5 mL 1X PBS, centrifuged and then lysed using RIPA lysis buffer (1M NaF, 1M β - glycerophosphate, 1M DTT, 0.2M NaVO_3 , 0.1M PMSF, 1mg/mL leupeptin, 1mg/mL pepstatin, 1mg/mL aprotinin, 100 μ M benzamide, 1M Tris, 0.05% SDS, 1% Triton X-100, 1% Igepal CA-630, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, pH 8.0, and 12 mM Na-Deoxycholate). Lysates were spun at 14,000rpm for 10 minutes to pellet the cell debris. The cleared lysates were then assayed for protein concentration using a Bradford Lowry Reagent (Bio-Rad, Mississauga, Ontario, Canada).

For western blotting, 40 μ g of protein was electrophoresed on an 8% polyacrylamide gel and transferred onto a PVDF (polyvinylidene difluoride) membrane (Thermo Fisher Scientific, USA). Membranes were blocked in 5% BSA (bovine serum albumin) (Sigma-Aldrich, Oakville, Ontario, Canada) in 1X TBST (50 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for one hour and primary antibodies were added in 5% BSA in 1X TBST for one hour at room temperature. Membranes were then washed in 1X TBST for 3x10 minutes. Reactive proteins were detected using horseradish peroxidase-coupled secondary antibodies (Bio-Rad, USA) and Western Lightning Plus enhanced chemiluminescence (Perkin-Elmer, USA). The following primary antibodies were used: anti-ZO-1 (Invitrogen, Camarillo, California), anti-E-cadherin (BD Transduction Laboratories, Canada), anti-SLK (custom antibody, Japan, (Wagner, et al., 2008)), anti- α -tubulin (Sigma-Aldrich, Oakville, Ontario, Canada), anti-Smad3 (Cell Signalling, USA), anti-phospho Smad3 (Cell Signalling, USA), anti-Smad2/3 (Cell Signalling, USA), anti-

LaminA/C (Cell Signaling, USA), anti-GAPDH (Abcam, USA), and anti-vimentin (Abcam, USA).

2.4 Immunofluorescence

Cells were plated onto cover slips contained within 6-well plates and grown overnight at 37°C in 5% CO₂ in growth medium. Prior to staining, the media was aspirated off the cell, and the cover slips were washed three times with 1X PBS (8mM Na₂HPO₄, 2mM NaH₂PO₄, 0.1 M NaCl, and 3 mM KCl). Coverslips were fixed with 1 mL of 4% PFA for 10 minutes, washed three times with 1X PBS, permeabilized with 0.1% Triton X in PBS for five minutes and washed again with 1X PBS. The coverslips were then blocked in 5% goat serum (Sigma-Aldrich, USA) in 1X PBS, washed, and incubated with primary antibody at room temperature. Antigens were detected using fluorescently labeled secondary antibodies. To stain for F-actin, a fluorescent conjugate of phalloidin was used (ThermoFisher Scientific, USA). Cover slips were then mounted onto microscope slides using a drop of ProLong Gold antifade reagent with DAPI (4, 6-diamidino-2-phenylindole) (Invitrogen, USA). Slides were sealed with nail polish and then visualized using a Zeiss AxioCam fluorescence microscope.

For cells infected with AdshSLK adenovirus, the cells were plated onto the cover slips 48 hours post infection. TGFβ-1 (Sigma-Aldrich, St. Louis, Montana, United States) treatments were as described in the figure legend at a concentration of 2ng/mL in serum-free media.

2.5 Transwell Migration and Invasion Assays

Prior to plating onto the migration chambers, cells were treated with either shSLK adenovirus or scramble adenovirus (as a control) for 48 hours, as described in section 2.2. The cells were treated with 2ng/mL of TGF-β for 48 hours and 5.0 x 10⁴ cells were plated into the top of each fibronectin-coated chamber of 8 μm pore, 24-well plates (Fisher Scientific, USA).

Haptotaxis assays were run with cells migrating in 0.2% FBS DMEM media, and chemotaxis assays were run with cells migrating from 0.2% FBS DMEM media at the top of the chamber to 0.2% FBS DMEM TGF- β -containing media at the bottom of the chamber. The migration chambers were placed at 37°C at 5% CO₂ for six hours. Each well was washed three times in PBS and fixed in 4% PFA for ten minutes. The wells were then washed and the membranes were removed and placed cell-side up onto a microscope slide. Each membrane was covered in ProLong Gold antifade reagent with DAPI (4, 6-diamidino-2-phenylindole) (Invitrogen) to coat the entire slide. The microscope slides were sealed with nail polish and visualized using a Zeiss AxioCam fluorescence microscope. Assays were done in triplicate wells and migrated cells were enumerated from five random fields of view per membrane. Invasion assays were performed as above but using Matrigel invasion chambers (BD Biosciences, Canada) and allowing cells 24 hours to chew through the matrigel.

2.6 Immunoprecipitation and Kinase Assays

NMuMG cells were plated at a concentration of 3.0×10^6 cells/ 10 cm plate and incubated for 24 hours. The cells were then serum-starved overnight and treated with 2ng/mL of TGF β in serum-free media for various times. Cells lysates were collected as described above and 400 μ g of protein was subjected to immunoprecipitation with anti-SLK antibodies (1 μ L) and 20 μ L of Protein A beads (GE Healthcare, USA). The mixture was left to rotate at 4°C for 2 hours and then washed three times in 1X NETN (20mM Tris pH 8, 1mM EDTA pH 8, 200mM NaCl, 0.5% NP-40, and water) and once in 1X kinase buffer (0.02M Tris pH 7.4, 0.001M NaF, 0.01M β -Glycerophosphate, 0.001M DTT, 0.015M MgCl₂, 250 μ M NaVO₃, and water). In vitro kinase assays were initiated by the addition of ³²P- γ ATP (~5 μ Ci, Perkin Elmer, USA) in 20 μ L of SLK kinase buffer. The reactions were incubated at 30°C for 30 minutes and terminated with the

addition of 7 μ L of 4X SDS sample buffer (0.2M Tris pH 7.4, 0.4M DTT, 8% SDS, 4mL glycerol, 6mM bromophenol blue). The samples were boiled for 5 minutes and loaded onto an 8% polyacrylamide gel, transferred onto a PVDF membrane and exposed to X-ray film.

2.7 RNA Extraction and Quantitative PCR Analysis

NMuMG cells were plated at 3 x 10⁶ cells per 10 cm plate and grown for 24 hours. The cells were then serum-starved overnight treated with 2ng/mL of TGF β 1 (Sigma-Aldrich, USA) for the indicated time points. Total RNA was extracted using Trizol (Ambion, Life Technologies, USA) as per the manufacturer's protocol. The final RNA pellets were washed with 1mL of 75% ethanol and air-dried. The RNA was re-suspended in RNase-free water for use in cDNA synthesis. To ensure no DNA contamination, the samples were run through the QIAGEN RNA clean-up kit (QIAGEN, USA) as indicated by the manufacturer.

For qPCR analysis, 5 μ g of total RNA was converted into cDNA using Superscript III Reverse Transcriptase (Invitrogen, USA) in a cocktail containing 0.5mM dNTP mix, and 250ng of oligo (dT)₁₂₋₁₈ (Invitrogen) in 1X First strand Buffer with 0.1M DTT (Invitrogen) and RNase OUT Recombinant RNase Inhibitor (Invitrogen). The cDNA was then added to a master mix of iTaq Universal SyBr Green Supermix (dNTPs, MgCl₂ and DNA polymerase) (Bio-Rad), primers and sterile water and run on a T100 Thermal Cycler (25°C for 5 minutes, 55°C for 60 minutes, and 70°C for 15 minutes). The qPCR was run in triplicate on a 96-well plate using three biological replicates per sample on an Applied Biosystems 7500 Fast Real-Time PCR System machine. The primer sequences used were: SLK, 5'-CTTCAGGCGCTTTGAGCAGG-3', and 5'-TTCTTGTTCCCTCCTTCTTGCGGT-3'; E-Cadherin, 5'-CTTCCGAAAAGAAGGCTGTCC-3' and 5'-CAGGTCTCCTCATGGCTTTGC-3'; Snail1, 5'-GTCAGCAAAGCACGGTTG-3' and 5'-CTTGTGTCTGCACGACCT-3', Snail2 5'-GATGTGCCCTCAGGTTTGAT-3' and 5'-

GGCTGCTTCAAGGACACATT-3', Vimentin, 5'-CACATCGATCTGGACATGCTGT-3' and 5'-CGGAAAGTGGAATCCTTGCA-3', Twist1, 5'-GGGACGCGGACATGGACC-3' and 5'-CACGCTGCCCTCGGACAA-3', Twist2, 5'-GTCATGAGGAGCCACAAGGT-3' and 5'-ATGTCCGCCTCCCCTAGC-3', Fibronectin, 5'-GCCCAGTGATTTTCAGCAAAGG-3' and 5'-ATGTGGACCCCTCCTGATAGT-3'.

2.8 Cell transfections

For transfection, NMuMGs were seeded at 3×10^6 cells on a 10cm plate and grown overnight to 70-90% confluency. The cells were transfected with Lipofectamine 3000™ Reagent in serum-free media, adjusted for a 10 cm plate according to the manufacturer's instructions. The transfection mix was then added to the adherent cells and topped up with 10% FBS DMEM after 3 hours. The cells were harvested 48 hours following transfection.

2.9 Subcellular Fractionation

Subcellular fractionation was used to effectively separate the nuclear and cytoplasmic fractions in NMuMG cells. The cells were grown to approximately 75% confluency on 10cm plates and washed once in 1X PBS and once in 1X PBS supplemented with 1mM EDTA (Ethylenediaminetetraacetic acid). Cells were collected using a cell scraper, and centrifuged at 1300 x g for 5 minutes to pellet the cells. The cells were lysed in the cytoplasmic lysis buffer (300mM sucrose, 20mM HEPES (pH 7.4), 0.5% NP-40, 50mM NaCl, 3mM MgCl₂, and a protein inhibitor cocktail (1M NaF, 1M β- glycerophosphate, 1M DTT, 0.2M NaVO₃, 0.1M PMSF, 1mg/mL leupeptin, 1mg/mL pepstatin, 1mg/mL aprotinin, 100μM benzamide, 1M Tris, 0.05% SDS, 1% Triton X-100, 1% Igepal CA-630, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, pH 8.0, and 12 mM Na-Deoxycholate)). The cells were rotated at 4°C for 10 minutes, and then centrifuged at 1300 x g at 4°C for five minutes. The cytoplasmic fraction was

collected and the pellet was re-suspended in the nuclear lysis buffer (20mM HEPES (pH 7.4), 1% NP-40, 25 mM NaCl, 1.5mM MgCl₂, and a protein inhibitor cocktail (1M NaF, 1M β-glycerophosphate, 1M DTT, 0.2M NaVO₃, 0.1M PMSF, 1mg/mL leupeptin, 1mg/mL pepstatin, 1mg/mL aprotinin, 100μM benzamide, 1M Tris, 0.05% SDS, 1% Triton X-100, 1% Igepal CA-630, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, pH 8.0, and 12 mM Na-Deoxycholate)). The suspension was rotated at 4°C for 10 minutes, and then centrifuged at 1300 x g for 5 minutes. The pellet, containing the nuclear fraction was re-suspended in a RIPA lysis buffer (1M NaF, 1M β- glycerophosphate, 1M DTT, 0.2M NaVO₃, 0.1M PMSF, 1mg/mL leupeptin, 1mg/mL pepstatin, 1mg/mL aprotinin, 100μM benzamide, 1M Tris, 0.05% SDS, 1% Triton X-100, 1% Igepal CA-630, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, pH 8.0, and 12 mM Na-Deoxycholate), and kept on ice for 20 minutes, vortexing every 5 minutes. Finally, the solution was centrifuged at 20, 000 x g at 4°C for 20 minutes, leaving the nuclear fraction in the supernatant. The lysates were run out on a gel and analyzed using western blotting as described in section 2.3.

3.0 RESULTS

3.1 SLK is present at the adherens junctions of mammary epithelial cells

Prior to investigating its role in EMT, the cellular distribution of SLK was examined in unstimulated NMuMG mammary epithelial cells. SLK was co-immunostained with both epithelial and mesenchymal cell markers to identify its localization within the cell. An experiment conducted with normal epithelial cells showed that in an unstimulated environment, SLK is found diffuse in the cytoplasm with increased reactivity at the cell-cell junctions, co-localizing with E-cadherin, an epithelial cell marker found in the adherens junctions (**Figure 7C**). Upon TGF β 1 treatment, SLK was re-distributed at the leading edge of migrating cells (**Figure 7D**). This has been previously observed in migrating fibroblasts (Wagner et al., 2008). We observed that E-cadherin downregulation is visible 48 hours following TGF β 1 treatment in normal murine mammary gland cells (**Figure 7E**). Fibronectin is a mesenchymal marker that is activated during EMT. It is a key extracellular ligand that binds integrins and is important in the formation of focal adhesions (Imamichi & Menke, 2007) (Zhao, Min, Vora, Trackman, Sonenshein, & Kirsch, 2008). Fibronectin expression was upregulated following TGF β 1 treatment (**Figure 7K**), suggesting that the mesenchymal transition was successfully induced by TGF β . Therefore, the addition of TGF β to NMuMG cells for a period of 48 hours was sufficient to induce the epithelial-to-mesenchymal process. The expected phenotypic changes were observed, as indicated by the upregulation of mesenchymal markers, and the downregulation of epithelial markers.

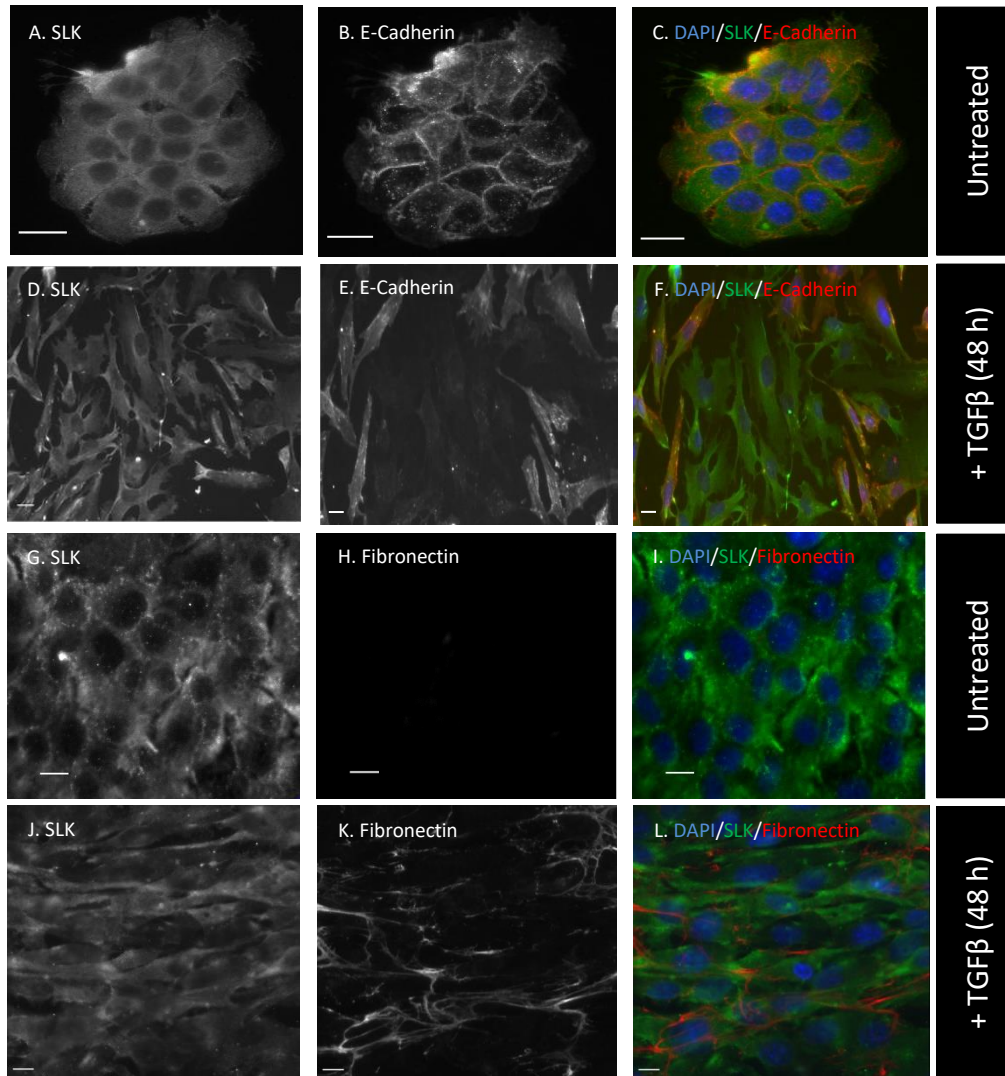


Figure 7. Localization of SLK in murine mammary epithelial cells following TGF β 1 stimulation. Immunofluorescence was performed on NMuMG cells in the presence or absence of 2ng/mL of TGF β 1 and stained for both epithelial and mesenchymal markers to observe the localization of SLK. Cells were co-stained with antibodies against SLK (green), E-Cadherin (red), and DAPI (blue) in panels **A-F**. In panels **G-L**, cells were co-stained with antibodies against SLK (green), fibronectin (red) and DAPI (blue). Scale bar represents 10 μ m and applies to all panels.

3.2 SLK knock down impairs TGF β -driven motility and invasion

To investigate the role of SLK in EMT progression, NMuMG cells were infected with GFP-tagged AdshSLK or a scrambled control prior to TGF β treatment. Cells were treated with the virus for at least 48 hours to ensure complete knockdown of SLK levels. Efficient SLK knock down was obtained at an MOI of about 10 and was validated by western blot analysis. Its expression was found to be undetectable approximately 48 hours after infection (**Figure 8A**) and was maintained for at least 96 hours (**Figure 8B**).

During EMT, cells transition from an apical-basal polarity to a front-back polarity (Thiery, 2002). Cells that are able to undergo EMT present with a more invasive and migratory phenotype (Thiery J. , Acloque, Huang, & Nieto, 2009) (Hanahan & Weinberg, 2000). To test if SLK plays a role in TGF β -induced cellular migration and invasion, NMuMG cells were first infected with either a scrambled control or shSLK GFP-tagged adenovirus for 48 hours to allow knockdown of SLK expression. The cells were then treated with TGF β for 48 hours to induce EMT (refer to **Figure 7**) and plated in a Boyden chamber to analyze cellular migration and invasion. In the absence of SLK, NMuMG cells showed a two-fold decrease in their migratory capacity towards a fibronectin-coated membrane following a haptotactic assay protocol (**Figure 9A&B**). Similarly, in a chemotaxis assay, the migration towards a gradient of TGF β was decreased by three-fold in the absence of SLK (**Figure 9C&D**). SLK knock down was previously shown to inhibit heregulin-driven invasion in breast cancer cells (Roovers et al., 2009). Therefore, the role of SLK in TGF β -induced invasion was also analyzed using matrigel-coated membranes. As observed in the haptotactic and chemotactic assays, SLK knockdown significantly impaired TGF β -driven cellular invasion. Enumeration of the cells showed a

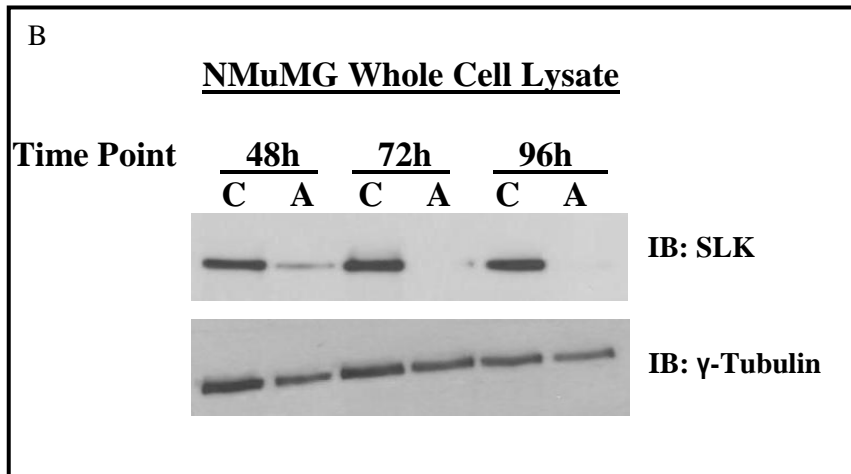
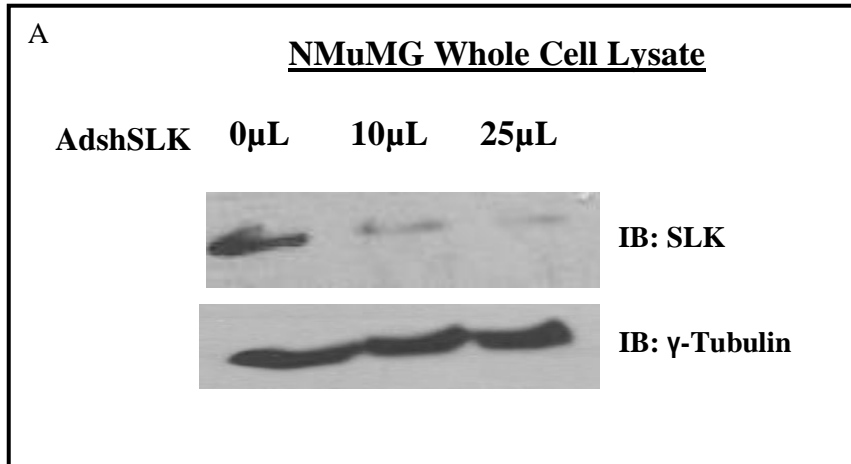


Figure 8. Optimization of SLK short hairpin adenovirus in NMuMG cells. (A) The SLK adenovirus was first optimized using increasing volumes of crude virus and collecting the lysate 48 hours following infection. (B) Next, the cells were infected with either AdGFP shSLK or AdGFP scramble virus to evaluate the length of time that knockdown can be sustained. These blots are representative data.
N=3. IB= Immunoblot; C=Control scramble adenovirus; A=SLK adenovirus.

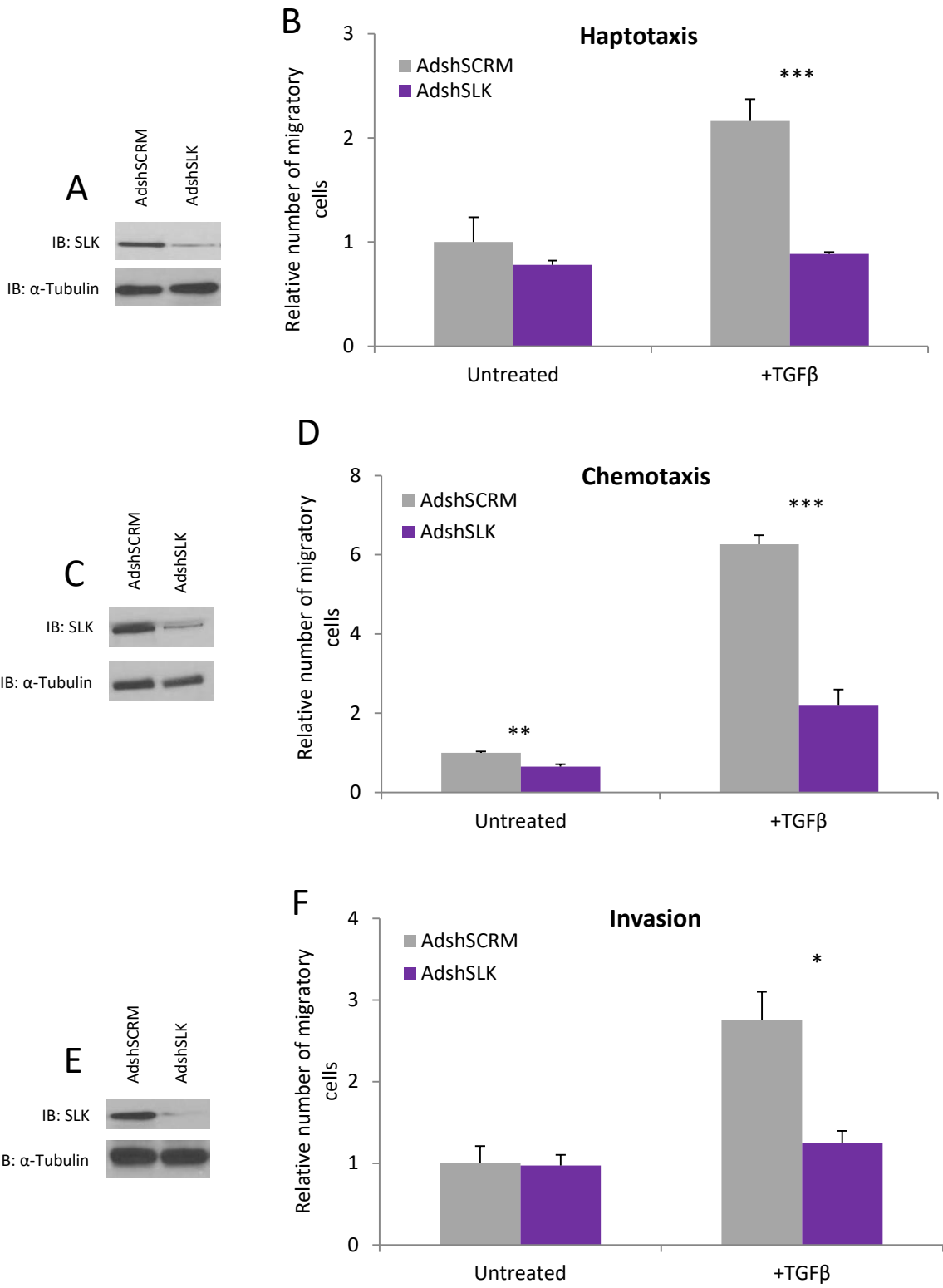


Figure 9. Migratory and invasive capabilities of NMuMG cells in the presence and absence of SLK following TGF β 1 treatment. (B) Haptotactic assay (D) Chemotactic assay, and (F) Invasion assay demonstrating relative number of cells migrating. Each experiment was completed in triplicate. Cells successfully migrated to the membrane were fixed in 4% PFA and counted using Image J software. Panels A, B, and C demonstrate corresponding western blots demonstrating SLK knockdown with antibodies against SLK and α -Tubulin. All assays were performed in triplicate and were counted relative to the untreated scramble control.
*p<0.05, **p<0.01, ***p<0.001

significant 2.5-fold reduction in the invasive capacity of NMuMG cells treated with TGF β in the absence of SLK (**Figure 9E&F**). This more strongly suggests that SLK plays a role in TGF β -dependent EMT as the invasion assay does not exclusively evaluate migratory capacity. Together these data strongly identify SLK as a key player in TGF β -driven migration and invasion in mammary epithelial cells.

3.3 Knock down of SLK impairs TGF β 1-driven EMT

Activation of EMT signaling results in dramatic morphological changes that lead to downregulation of the epithelial cell markers E-cadherin and ZO-1 (**Figure 7**) (Miettinen, Ebner, Lopez, & Derynck, 1994). Additionally, actin changes from a cortical shape located at the cell-cell junctions to a polymerized stress fibre shape that stretches the length of the cell (Thiery, 2002). Therefore, to further characterize the role of SLK in TGF β -induced EMT, we investigated the effect of SLK knock down on the morphological changes associated with EMT in NMuMG cells. The cells were first infected with the scramble control or shSLK GFP-tagged adenovirus to knockdown SLK expression. The cells were then treated with 2ng/mL of TGF β 1 for 48 hours to induce EMT and immunostained with various markers. Supporting the motility and invasion data, we observed a marked inhibition in the breakdown of ZO-1-positive tight junctions (**Figure 10G-H**) and E-cadherin-positive adherens junctions (**Figure 10S-T**). In addition, phalloidin staining revealed that SLK knock down also inhibited the cytoskeletal changes associated with the mesenchymal phenotype (**Figure 10I-J**). Whereas the control cells showed an elongated fibroblast-like morphology (**Figure 10C-D**), the SLK-depleted cells maintained an epithelial morphology. Together, these data suggest that SLK is required for the morphological changes that occur during TGF β -induced EMT.

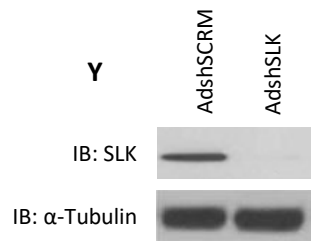
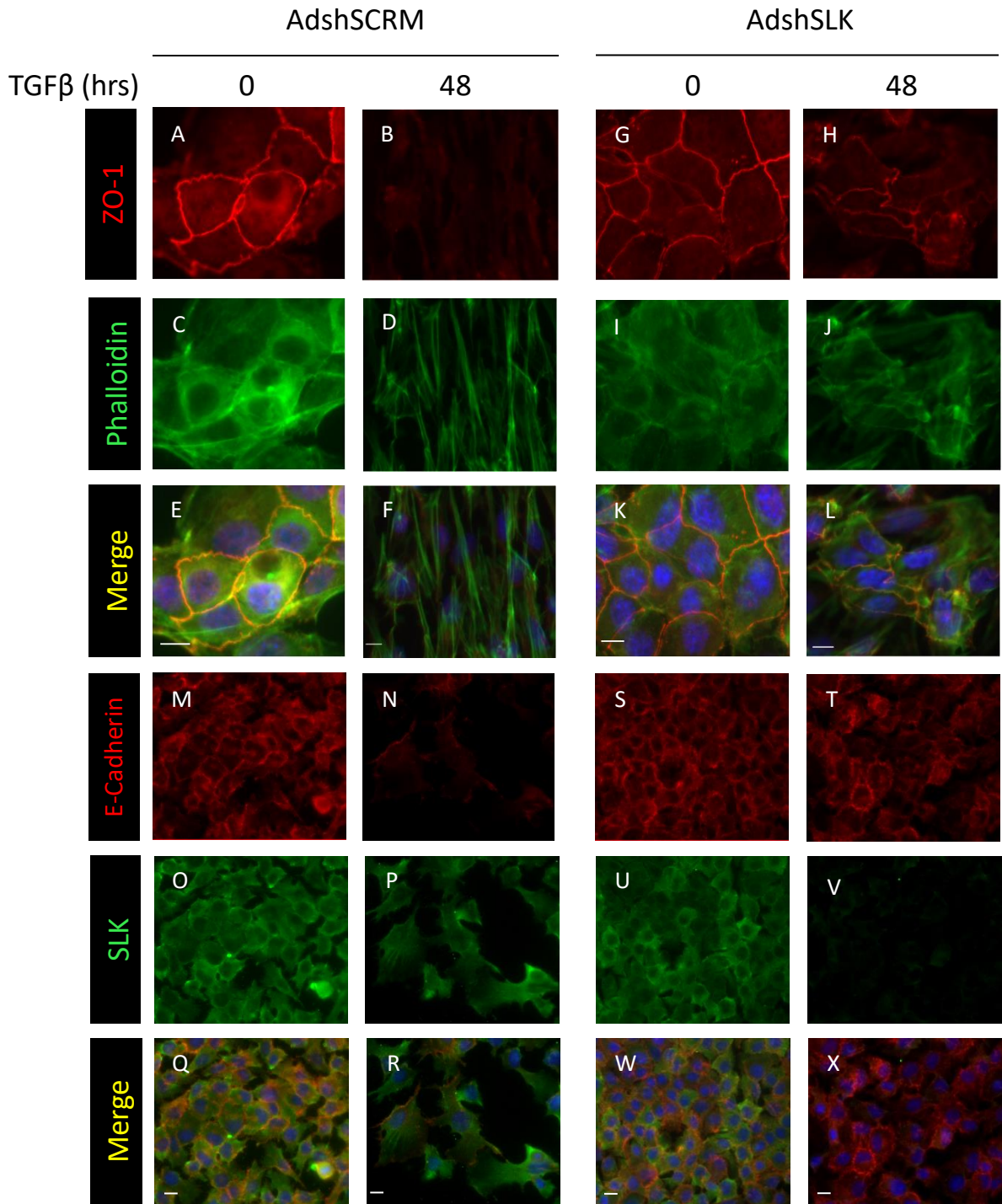


Figure 10. In the absence of SLK, TGF β 1-treated NMuMG cells maintain an epithelial phenotype. NMuMG cells were treated with AdshSCRM and co-stained with ZO-1 (red), actin (green), and DAPI (blue) in panels **A-F**. Cells were treated with AdshSLK and co-stained with antibodies against ZO-1 (green), actin (red) and DAPI (blue) in panels **G-L**. Cells were treated with AdshSCRM and co-stained with antibodies against SLK (green), E-Cadherin (red) and DAPI (blue) in panels **M-R**. Finally, cells were treated with AdshSLK and co-stained with antibodies against SLK (green), E-Cadherin (red) and DAPI (blue) in panels **S-X**. The corresponding western blot demonstrating SLK knockdown is shown in panel **Y**. Scale bar represents 10 μ m and applies to all panels. N=3.

3.4 SLK knock down does not affect the phosphorylation or translocation of the R-Smads

The canonical signaling pathway in EMT involves multiple dimeric proteins called Smads that participate in a signaling cascade that ultimately results in both cytoskeletal and genetic changes to the cell (Mehra & Wrana, 2002). Smads 2 and 3 get phosphorylated by the TGF β R complex and form a trimer with the Smad 4 protein. This complex then translocates into the nucleus and binds to DNA to exert its effects through transcriptional activation of mesenchymal markers and transcriptional repression of epithelial markers (Derynck & Zhang, 1996). To further investigate the role of SLK downstream of TGF β signalling, the activation status of the canonical Smad-dependent pathway was assessed in the absence of SLK.

To begin our analysis, we first explored the effect of SLK knockdown on receptor-Smad phosphorylation and protein stability. We first knocked down SLK expression in NMuMG cells and treated the cells with 2ng/mL of TGF β 1 for either 0, 1 or 24 hours to stimulate the EMT process. Western blot analysis shows that, in the absence of SLK, the levels of phospho Smad3 or total Smad3 are unaffected, suggesting that SLK depletion does not block the rapid activation of the Smads by the receptor (**Figure 11A**).

The Smad proteins must translocate into the nucleus to drive gene transcription associated with the EMT response (Whitman, 1998) (Massague J. , 1998) (Lagna, Hata, Hemmati-Brivanlou, & Massague, 1996) (Macias-Silva, Abdollah, Hoodles, Pirone, Attisano, & Wrana, 1996). One possibility is that SLK knock down impairs the translocation of the Smad complex to the nucleus, preventing the downregulation of E-cadherin. Therefore, to assess this we performed immunofluorescence and cellular fractionation studies. SLK was knocked down in NMuMG cells for 48 hours and the cells were treated with 2ng/mL of TGF β 1 for either one or two hours. Following this, cytoplasmic and nuclear fractions were analysed for Smad3

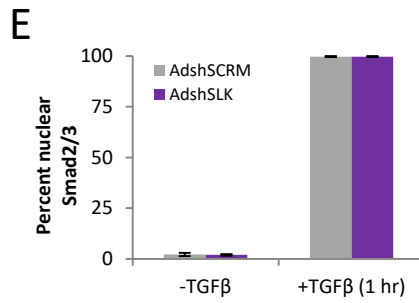
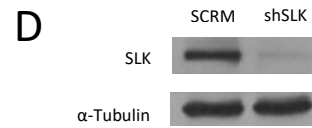
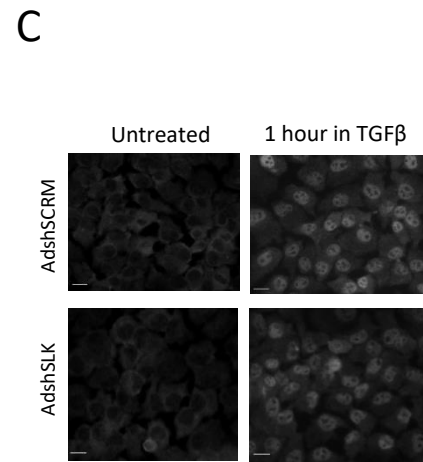
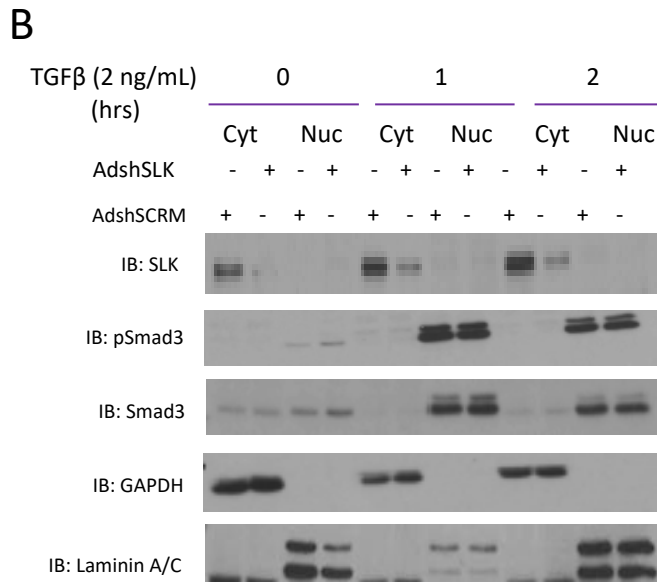
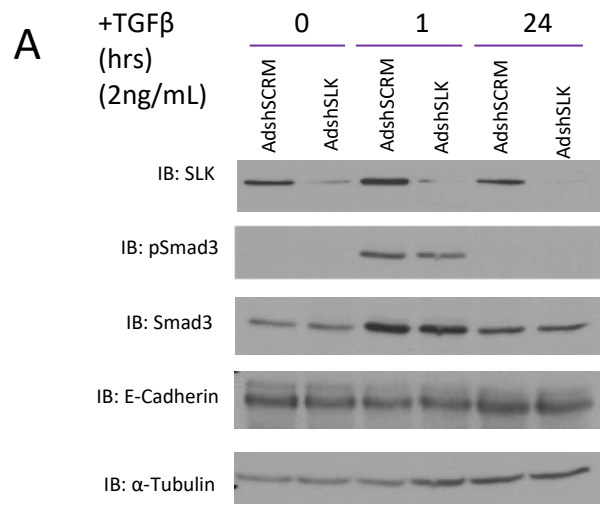


Figure 11. SLK does not affect the phosphorylation or translocation of the R-Smads. (A) NMuMG cells were first infected with AdshSCRM or AdshSLK for 48 hours prior to treatment with 2ng/mL of TGF β 1 for 1 or 24 hours. Analysis was done by immunoblotting using antibodies against SLK, pSmad3, Smad3, E-Cadherin, and α -tubulin. (B) A subcellular fractionation was completed in NMuMG cells to effectively separate the cytoplasmic and nuclear fractions. Analysis was done by immunoblotting using antibodies against SLK, pSmad3, Smad3, GAPDH, and LaminA/C. (C) NMuMG cells were infected with AdshSCRM or AdshSLK for 48 hours prior to TGF β 1 treatment for one hour. Immunofluorescence was completed using antibodies against total Smad2/3. Scale bar represents 10 μ m. (D) Western corresponding to 4D to demonstrate SLK knockdown using antibodies against SLK and α -Tubulin. (E) Quantification of Smad2/3 nuclear expression relative to nuclear DAPI staining corresponding to the immunofluorescence panel in 4D. Error bars represent SE, N=3 for all experiments.

expression and validated with a lamin A/C and GAPDH as a nuclear and cytosolic marker, respectively. Our results show that phospho Smad3 was capable of nuclear translocation, suggesting that SLK depletion does not impair the shuttling of the Smad2/3/4 transcriptional complex (**Figure 11B**). Supporting this, immunofluorescence staining for the Smad2/3 complex revealed no significant differences in the number of Smad2/3 positive nuclei between the AdshScramble and AdshSLK-treated cells (**Figure 11C-E**). This suggests that, in the absence of SLK, the R-Smads 2 and 3 are capable of translocating to the nucleus upon TGF β 1 stimulation. Together these data suggest that Smad activation through the canonical pathway is unaffected by SLK depletion.

3.5 SLK is required for the induction of a subset of TGF β -regulated genes during EMT

Following TGF β 1 treatment in NMuMG cells, epithelial markers become downregulated and the expression of mesenchymal markers is induced (Xu, Lamouille, & Derynck, 2009). Therefore, to gain further insights into the role of SLK in TGF β -mediated EMT we investigated the genetic response downstream of TGF β treatment. We performed qPCR to evaluate the mRNA levels of target genes shown to be up or down-regulated following TGF β stimulation. We first knocked down SLK for 48 hours and treated the cells with TGF β 1 at a concentration of 2ng/mL. Total RNA was extracted every 3 hours for 15 hours and subjected to qPCR analysis. A significant knockdown of SLK was achieved at the level of both the mRNA and protein across all time points (**Figure 12C**). We first surveyed the expression of Snail1, a mesenchymal transcriptional target that is upregulated following TGF β stimulation (Kaufhold & Bonavida, 2014). Interestingly, our results suggest that in the absence of SLK, Snail1 expression was

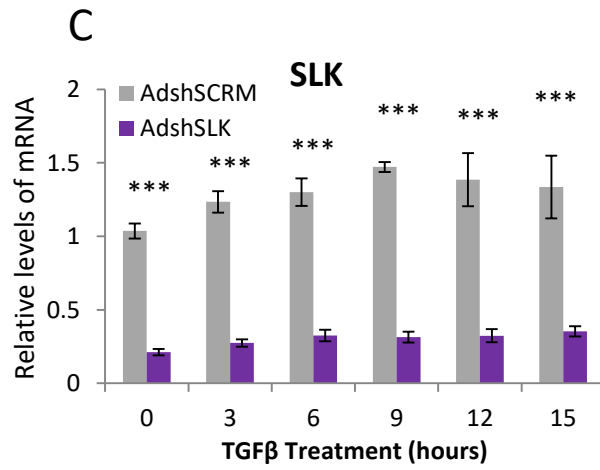
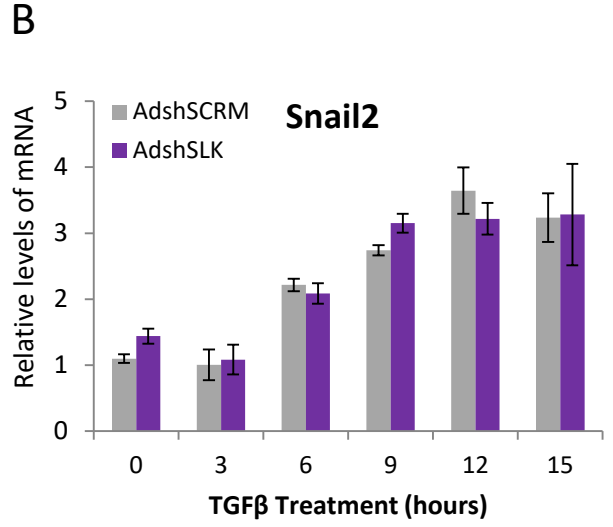
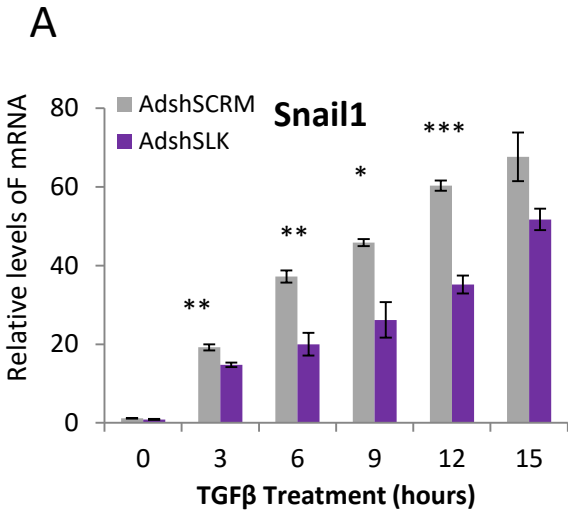


Figure 12. SLK knockdown conditions significantly affect Snail1 mRNA levels following short-term TGF β treatment. RNA was extracted from NMuMG cells and mRNA levels were evaluated using qPCR. Primers were used to evaluate (A) Snail1 at 0,3,6,9, 12 and 15 hours, (B) Snail2 at 0,3,6,9, 12 and 15 hours, (C) SLK at 0,3,6,9, 12 and 15 hours. p<0.05, **p<0.01, ***p<0.001, Error bars represent SE, N=3 for all experiments.

significantly reduced throughout the time course compared to the scrambled control sample (**Figure 12A**). Surprisingly, Snail2 (Slug) expression was not changed significantly in the absence of SLK (**Figure 12B**). This suggests that SLK downregulation impairs the transcriptional activation of a subset of targets, likely resulting in an impaired EMT response.

We continued our qPCR analysis at longer time points to look for differences in other downstream targets that are known to be affected by Snail1 expression levels. Evaluation of a mesenchymal target, fibronectin, demonstrated that its expression was significantly reduced by 41% over 12 hours (**Figure 13A**). Analysis for E-cadherin expression, a key epithelial marker, demonstrates that its expression was significantly higher following a 24-hour TGF β treatment in the absence of SLK (**Figure 13B**), suggesting that its complete downregulation is also impaired in the absence of SLK. Notably, prior to TGF β treatment, the E-cadherin levels in the SLK-depleted cells are also higher than control. Vimentin is a key mesenchymal marker upregulated in TGF β -dependent EMT (Xu, Lamouille, & Derynck, 2009). Western analysis showed that SLK knock down suppressed vimentin expression two days following TGF β treatment (**Figure 13D**). SLK knockdown was also demonstrated in all mRNA samples (**Figure 13C**).

3.6 SLK kinase activity is unaffected by TGF β stimulation

As SLK is present at cell-cell junctions and its depletion impairs TGF β -driven motility and invasion, we tested whether TGF β stimulation induces its kinase activity. NMuMG cells were serum-starved and treated with TGF β at increasing time points. The cells were harvested, lysed and subjected to *in vitro* kinase assays. Whereas pSmad3 was induced within 10 minutes of TGF β stimulation, kinase assays showed that SLK activity was not modulated by following stimulation with TGF β 1 during a short time course (**Figure 14**). Similarly, longer time courses

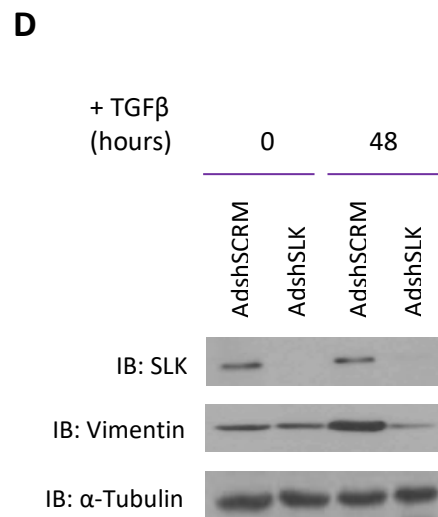
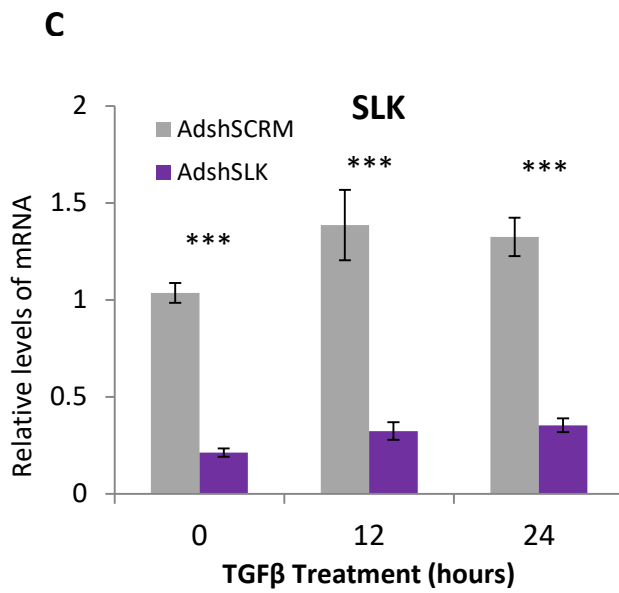
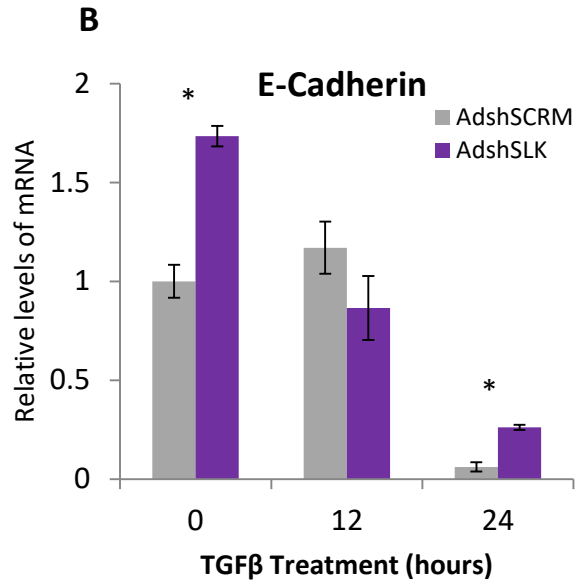
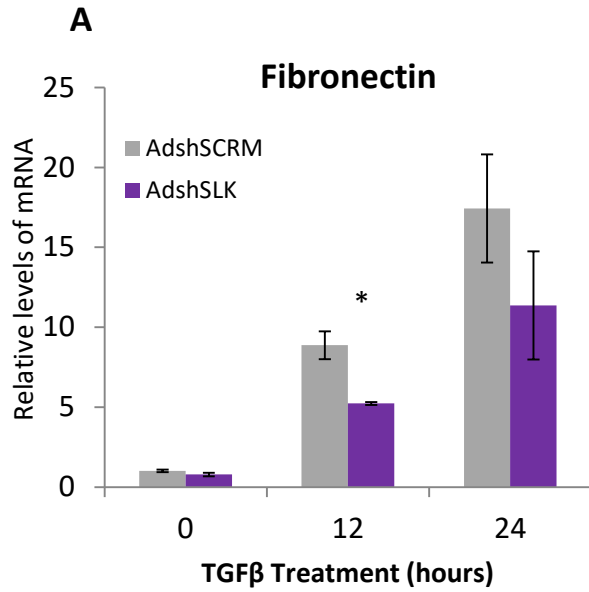


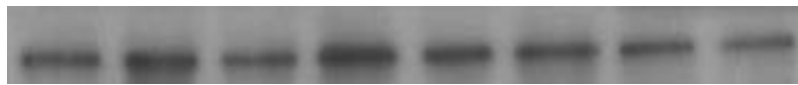
Figure 13. SLK knockdown conditions significantly affect fibronectin and E-cadherin mRNA levels and vimentin protein levels following long-term TGF β treatment. RNA was extracted from NMuMG cells and mRNA levels were evaluated using qPCR. Primers were used to evaluate (A) fibronectin at 0, 12 and 24 hours, (B) E-cadherin at 0, 12 and 24 hours, and (C) SLK at 0,12 and 24 hours. (D) In the western blot analysis, we infected NMuMG cells as described above, and treated with TGF β for 0 and 48 hours. Blots were analyzed using antibodies against SLK, vimentin, and α -tubulin.

*p<0.05, **p<0.01, ***p<0.001, error bars represent SE, N=3 for all experiments.

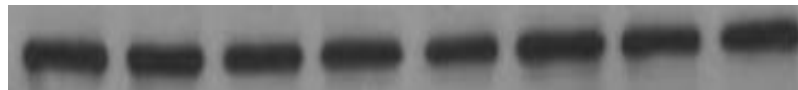
TGFβ (min)
(2ng/mL)

0 5 10 15 20 25 30 35

AutoRad
IP: SLK



IP: SLK
IB: SLK



IP: SLK

WCL
IB: SLK



WCL
IB: pSmad3



WCL
IB: Smad3



WCL
IB: Actin



Whole
cell
lysate

Figure 14. SLK kinase activity is unaffected downstream of the TGF β receptor complex. A kinase assay was performed using NMuMG cells which were treated with 2ng/mL of TGF β 1 at various time points. The lysates were immunoprecipitated with an antibody specific to SLK and were exposed to ^{32}P - γ ATP to evaluate SLK kinase activity.
IP= immunoprecipitate, IB= immunoblot, WCL= whole cell lysate, N=3.

showed no activation of SLK (not shown). This suggests that TGF β stimulation does not activate SLK, and it likely plays a kinase-independent role.

3.7 SLK regulates EMT in a kinase activity independent manner

As TGF β stimulation does not modulate SLK kinase activity, one likely possibility is that SLK affects EMT signaling through a scaffolding function. To test this, a kinase-dead version of SLK (K63R) was expressed into NMuMG cells at high levels. Activation of SLK occurs through homodimerization in a *trans* orientation, resulting in autophosphorylation and activation (Delarosa, Guillemette, Papillon, Han, Kristof, & Cybulsky, 2011) (Pike, et al., 2008). A point mutation at lysine 63 to an arginine (hereby referred to as K63R) inactivates the full-length kinase but still allows dimerization with endogenous SLK to inactivate the entire complex (Sabourin, Tamai, Seale, Wagner, & Rudnicki, 2000). Immunoprecipitation and kinase assays of transfected SLK or K63R into NMuMG cells shows that expression of K63R markedly reduces the autophosphorylation signal (**Figure 15A**). To test if K63R could impair TGF β -driven EMT, we surveyed the expression of Snail1 following transfection. NMuMG cells were transfected with either wild-type SLK or the K63R plasmid, and treated with TGF β for 9 hours. AdshScramble and AdshSLK-infected cells were used as controls. A qPCR analysis was performed with primers against Snail1 and SLK (**Figure 15B and C**). Interestingly, our results indicate that in the presence of K63R, total SLK activity is downregulated but Snail1 mRNA is upregulated to the same extent as the control plasmid and AdshScramble samples. The AdshSLK control sample showed a 50% reduction in Snail1 mRNA as expected. These data strongly suggest that SLK plays a kinase activity independent role in TGF β -induced EMT.

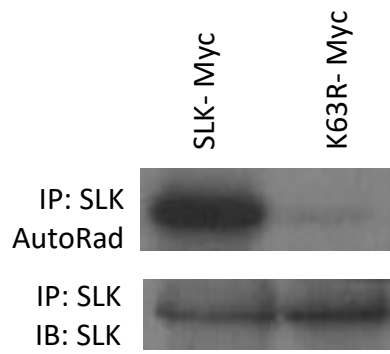
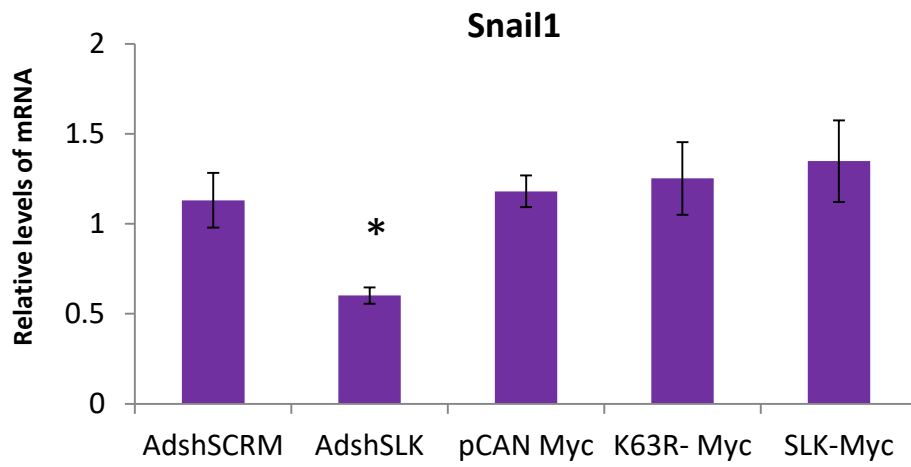
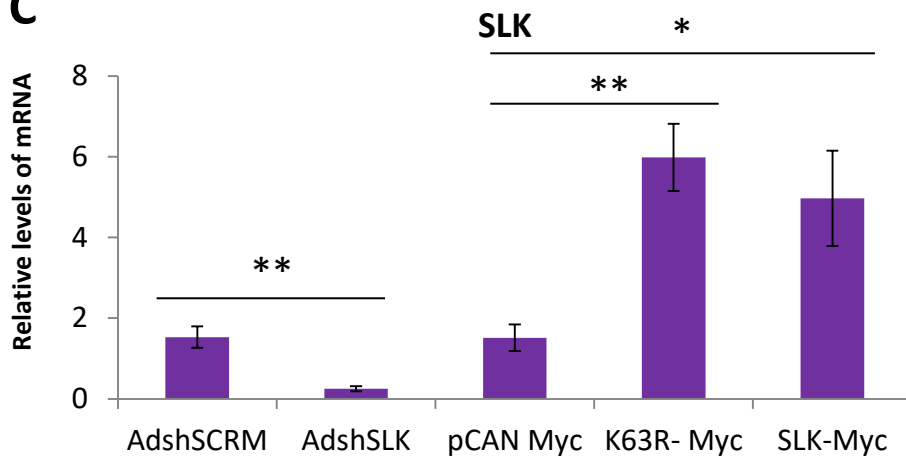
A**B****C**

Figure 15. SLK likely plays a scaffolding role in TGF β -mediated EMT. (A) NMuMG cells were transfected with Myc-SLK or Myc-K63R for 48 hours. Results were analyzed using western blotting with an antibody against SLK. (B, C) NMuMG cells were first infected with either AdshSCRM or AdshSLK for 48 hours. Next, cells were transfected with Myc-tagged pCan, K63R, and SLK for 48 hours. The cells were then serum-starved overnight and treated with 2ng/mL of TGF β 1 in serum-free media for 9 hours the following day. RNA was extracted mRNA levels were quantified using qPCR with primers against Snail1 (B) and SLK (C). Each experiment was run in triplicate with three biological replicates. Error bars represent SE. *p<0.05, **p<0.01, N=3 for all experiments.

4.0 DISCUSSION

4.1 SLK is recruited to the leading edge of migratory cells

Statistics demonstrate that 90% of cancer mortalities result from cancer progression that leads to an invasive and metastatic disease (Sporn, 1996) (Hanahan & Weinberg, 2000). Therefore, current research is attempting to understand the complex signaling pathways involved in cancer metastasis in an effort to reduce cancer-related deaths. One of the key signaling pathways involved in metastasis is epithelial-to-mesenchymal transition (EMT), a normal signaling pathway that is hijacked in cancer cells (Miettinen, Ebner, Lopez, & Derynck, 1994). Cells undergoing EMT lose expression of epithelial markers (E-cadherin), gain expression of mesenchymal markers (vimentin), and dramatically change into an elongated motile phenotype due to the polymerization of actin fibres (Huber, Kraut, & Beug, 2005).

Our lab has previously demonstrated that SLK is recruited to the leading edge of migratory cells, specifically within podosome-like cellular extrusions (Sabourin & Rudnicki, 1999) (Wagner, Flood, O'Reilly, Hume, & Sabourin, 2002). It has also been documented that SLK co-localizes with paxillin at the leading edge of migratory cells following scratch-wounding to stimulate migration, supporting the belief that SLK plays an important role in directing cell movement (Quizil, et al., 2013). Additionally, our lab has shown that SLK is involved in FA turnover during cellular migration through the disassembly of microtubules (Wagner, et al., 2008). Therefore, the presence of SLK at the leading edges of migratory cells supports a role for SLK in migration.

To investigate the role of SLK in epithelial cells, we used a normal murine mammary gland epithelial cell line called NMuMG. This is a non-transformed immortalized cell line that is estrogen receptor negative, progesterone receptor negative, and has negligible levels of HER2.

NMuMG cells were chosen to perform all experiments because they are extremely responsive to TGF β , meaning that they would be an excellent cell line in studying EMT (Miettinen, Ebner, Lopez, & Derynck, 1994) (Piek, Moustakas, Kurisaki, Heldin, & Dijke, 1999). Unfortunately, due to the low levels of HER2 in this cell line, our results will not provide any links to HER2 and SLK crosstalk in a TGF β -dependent EMT context. In NMuMG cells stimulated with TGF β , SLK localizes at the cell-cell junctions with E-cadherin, suggesting that SLK might be involved in adherens junctions stability (**Figure 7C**). Our data also demonstrates that E-cadherin expression is visibly downregulated beginning 48 hours following TGF β treatment (**Figure 7E**). In addition, TGF β treatment showed an increase in fibronectin, a key mesenchymal marker, indicating a successful transition towards EMT (**Figure 7L**). Our results suggest that SLK responds to TGF β -induced cellular migration by moving to the leading edge of migratory cells, upregulating key mesenchymal markers, and downregulating key epithelial markers.

4.2 Loss of SLK expression reduces the migratory and invasive capacity of cells

SLK activity is significantly increased following heregulin stimulation in migratory cells that overexpress HER2 (Roovers, et al., 2009). Additionally, our lab has shown that SLK phosphorylates paxillin, leading to focal adhesion turnover during cell migration (Quizzi, et al., 2013). We have also demonstrated that SLK is activated downstream of FAK/c-src signaling, which is required for microtubule-dependent focal adhesion turnover (Wagner, et al., 2008). Our rationale for investigating SLK in EMT signaling originates from these observations, linking SLK to cellular migration and HER2 signaling. To assess the role of SLK in TGF β -induced EMT we used NMuMG mammary epithelial cells that undergo rapid EMT following TGF β treatment (Miettinen, Ebner, Lopez, & Derynck, 1994). To evaluate TGF β -dependent EMT in

the absence of SLK, we chose to use a short hairpin adenovirus as our knockdown method. Our results demonstrate that we were able to reduce SLK expression by >90% (**Figure 8A**) up to 96 hours post infection (**Figure 8B**).

To evaluate the migratory capacities of NMuMG cells in the absence of SLK, we used Boyden chamber assays. It was observed that SLK knockdown dramatically reduced the number of cells that could successfully migrate towards a fibronectin-coated membrane or a TGF β gradient in haptotaxis and chemotaxis assays respectively (**Figures 9B & 9D**). To further evaluate the effect of SLK on EMT, we used an invasion assay that requires cells to breakdown a matrigel coating that simulates the extracellular matrix. The results from this assay demonstrated that SLK is required for TGF β -dependent cellular invasion (**Figure 9F**). Together, these results suggest that SLK plays a major role in a cell's migratory and invasive abilities downstream of TGF β -induced EMT.

One likely explanation is that the observed defect in migration results from a delay or block in the internal EMT signaling pathway. Previous studies in our lab have already demonstrated that SLK is directly involved in focal adhesion turnover during cellular migration (Wagner, et al., 2008) (Quizi, et al., 2013). The decrease in migration supports previous research which demonstrates that FA turnover is affected by the absence of SLK at the leading edge. It is unclear whether the reduction in migration is due to a defect in the EMT signaling pathway or in the focal adhesion turnover during cellular migration.

In a chemotaxis assay, our results demonstrate a similar defect. Under SLK knockdown conditions, the migratory ability of cells is reduced by at least twice that of the controls (**Figure 9D**). As discussed above, this defect could also be attributed to either a dysfunction in the EMT signaling pathway that prevents cells from transitioning into a migratory phenotype, or impaired

focal adhesion turnover required for cellular migration. Similarly, a defect in the invasive capacity (2.5-fold reduction) was also observed in the absence of SLK (**Figure 9F**). This was also shown in heregulin stimulated breast cancer cells (Roovers et al., 2009)

During invasion, the cells must first “digest” the matrigel coating by upregulating the expression of MMPs. However, we were unable to detect any significant differences in MMP9 expression up to 24 hours post TGF β treatment relative to controls (not shown), suggesting that the defect is upstream of MMP gene induction. However, one of the limitations of this mRNA analysis is the length of time cells were exposed to TGF β 1. Longer time course may reveal differences in MMP9 expression levels in the absence of SLK. One study has shown that MMP9 mRNA expression levels increased by 30-fold when comparing 24 hour and 48 hour time points (Lamouille, Connolly, Smyth, Akhurst, & Derynck, 2012). If we extended our mRNA analysis to 48 hours, we could potentially explain why shSLK-treated cells are unable to chew through the matrigel, thus reducing the number of cells that can reach the membrane at the bottom of the well.

4.3 The epithelial phenotype is maintained in the absence of SLK

Our results from the migration and invasion assays demonstrate that SLK appears to play an important role in cellular migration and invasion in TGF β -treated cells. With this data, we wanted investigate the possibility that SLK was somehow involved in the EMT signaling pathway and required for the expression of key mesenchymal markers. In untreated conditions, the E-Cadherin is located in the adherens junctions with a clear cobblestone appearance characteristic of epithelial cells (**Figure 10M**), and loses all cell-cell contacts in the presence of TGF β (**Figure 10N**). Using immunofluorescence experiments on SLK-depleted cultures we have

shown that the characteristic cell-cell junction breakdown was inhibited. This was apparent from the distinct localization of E-cadherin (**Figure 10T**) and ZO-1 (**Figure 10H**) at those junctions in the absence of SLK. Similarly, phalloidin staining revealed that SLK-depleted cells remained more epithelial and did not undergo the characteristic morphological changes of EMT (**Figure 10J**). These results suggest that SLK is required for the cytoskeletal changes induced by TGF β treatment and are unable to fully transition into a mesenchymal phenotype (Xu, Lamouille, & Derynck, 2009). These data provide evidence that SLK is required for signalling processes downstream of EMT activation.

Together with our migration data, one possibility is that SLK-depleted cells maintain an epithelial phenotype with decreases in migratory and invasive capacities. E-Cadherin expression at the adherens junctions has been shown to prevent the cell from transitioning into an invasive phenotype (Frixen, et al., 1991). However, we still cannot rule out the possibility that the cells are also unable to migrate due to an impaired focal adhesion turnover signaling pathway in the absence of SLK. It is likely that the resulting decreases in migration and invasion are a result of both dysregulated FA turnover, and a dysfunction in EMT cytoskeletal changes.

4.4 SLK does not affect Smad phosphorylation or localization

To further understand the role of SLK in EMT, we first explored its involvement in the Smad-dependent pathway. It has been well documented that the receptor Smads are directly phosphorylated by TGF β RI once the cells are treated with the TGF β ligand (Eppert, et al., 1996) (Nakao, Roijer, Imamura, Souchelnytskyi, Stenman, & Heldin, 1997b) (Yingling, Das, Savage, Zhang, Padgett, & Wang, 1996). We hypothesized that SLK could affect R-Smad phosphorylation or stability, thus preventing downregulation of E-Cadherin.

To study the effect on SLK deletion on the Smad proteins, we used western blotting, immunofluorescence, and subcellular fractionation techniques. Our results indicate that SLK does not affect Smad3 phosphorylation or total protein levels at one hour following TGF β treatment (**Figure 11A**). As previously reported, the Smad3 proteins were downregulated 24 hours after treatment to inactivate the pathway (Hayashi, et al., 1997) (Imamura, et al., 1997) (Nakao, Roijer, Imamura, Souchelnytskyi, Stenman, & Heldin, 1997b). One limitation of this experiment is the lack of Smad2 immunoblotting to demonstrate full activation of the receptor Smads. Since both Smad proteins are phosphorylated by the receptor complex, we believe that Smad3 immunoblotting is sufficient to show that TGF β was added to the cells.

Another critical aspect of Smad-dependent signaling is the translocation of the Smad2/3/4 complex into the nucleus (Baker & Harland, 1996) (Hoodless, et al., 1996) (Mehra & Wrana, 2002). To determine if Smad nuclear translocation was affected in the absence of SLK, we performed a sub-cellular fractionation that separated lysates into cytoplasmic and nuclear fractions. Our results indicate that Smad3 was capable of entering the nucleus following TGF β 1 treatment in both the scramble and AdshSLK-infected conditions (**Figure 11B**). Together, the data demonstrates that the Smad proteins are activated by phosphorylation and translocate to the nucleus in the absence of SLK.

As an additional test, we performed an immunofluorescence experiment to detect Smad2/3 nuclear translocation following TGF β 1 treatment. Our results indicate that in the absence of SLK, the Smad2/3 proteins can effectively enter into the nucleus as shown in **Figure 11C**. This further supports the conclusion that SLK does not alter receptor Smad phosphorylation or localization. The next question was to analyze the effects of SLK deletion on genetic transcription, another requirement in EMT signaling.

4.5 SLK significantly affects gene targets following TGF β treatment

Our data indicates that SLK depletion has no effect on Smad2/3 nuclear translocation or Smad3 phosphorylation, suggesting that SLK does not directly affect the activation of the Smad proteins. However, the Smad proteins have been shown to interact with many other transcriptional regulators within the cell (Mehra & Wrana, 2002). There are many pathways that can crosstalk with the Smad proteins downstream of the TGF β receptor complex, including Ras/Erk/p38 MAPK, PI3K/Akt and Rho-like GTPase pathways (Xu, Lamouille, & Derynck, 2009). These pathways are involved in key transcriptional events that are necessary for the cytoskeletal and genetic changes that occur during the EMT signaling pathway.

To further investigate the EMT defect imparted by SLK depletion, we analysed the mRNA levels of known EMT targets that are either upregulated (mesenchymal markers) or downregulated (epithelial markers) in presence of TGF β . To test this, we used quantitative PCR to evaluate mRNA levels and western blots to analyze protein levels following TGF β 1 treatment in NMuMG cells. Our results indicate that in the absence of SLK, Snail1 levels are significantly decreased at 3, 6, 9, and 12, and 24 hours following TGF β 1 treatment by 23.1%, 46.3%, 42.9%, 41.7%, and 44.3% respectively (**Figure 12A**). It is interesting to note that at 24 hours following TGF β stimulation, the Snail1 mRNA levels decrease significantly in comparison to the levels measured at 15 hours. This is an important result because the samples treated with the SLK adenovirus do not catch up to the scramble-treated control, and remain significantly lower throughout the TGF β treatment. These results indicate that it is more likely that the SLK-deleted cells experience a block in the Snail1 genetic response. This provides the first evidence that SLK plays a role in gene regulation. However, it is important to note that this observed change in Snail1 levels does not necessarily correlate to a change in the protein levels. Therefore, we

cannot conclude based on this data that Snail1 protein levels are also changing. We need to look further into Snail1 and its gene targets to better understand how a decrease in Snail1 mRNA levels affects our phenotype. Snail1 is an important transcription factor required for downregulation of E-Cadherin expression and upregulation of multiple mesenchymal markers including vimentin and fibronectin (Cano, et al., 2000). This observed decrease in Snail1 expression supports the immunofluorescence data demonstrating a prolonged E-cadherin expression, and a decrease in migration and invasion of NMuMG cells. A decrease in the availability of Snail1 would directly result in a decrease in the repression of E-cadherin expression.

Another member of the Snail family, called Snail2 (Slug) is also upregulated downstream of TGF β signaling (Romano & Runyan, 2000). Surprisingly there was no significant difference in Snail2 mRNA levels (**Figure 12B**). These results indicate that SLK depletion affects distinct EMT responses.

It has been well documented that Snail1 is critical for silencing E-Cadherin gene expression by binding directly to the E-box (Cano, et al., 2000). We demonstrated using quantitative PCR that the shSLK-treated cells express higher E-Cadherin levels prior to TGF β treatment, suggesting that SLK plays a role in E-Cadherin expression in normal conditions. We have also shown that in the absence of SLK, E-Cadherin levels remain higher than the scramble-treated cells after TGF β 1 treatment (**Figure 13B**). Considering that E-Cadherin expression is regulated, in part, by the transcription factor Snail1, it is possible that the failure to downregulate E-cadherin is due to the partial activation of Snail1 expression.

Snail1 is also an important transcription factor responsible for upregulating vimentin, fibronectin, and MMP9 (Cano, et al., 2000). Supporting a reduction in Snail1 mRNA levels, we

observed a significant difference in fibronectin mRNA levels and vimentin protein in the absence of SLK (**Figure 13A, 13D**). This data further supports the hypothesis that SLK affects the genetic component of TGF β -induced EMT, possibly through Snail1 induction. The reduced levels of fibronectin and vimentin are likely to affect the transition into a mesenchymal phenotype. The molecular mechanisms that regulate SLK function during EMT remain to be investigated.

4.6 SLK kinase activity is unaffected downstream of TGF β stimulation

Although SLK depletion affects the process of EMT, our results demonstrate that SLK activity is not modulated following stimulation with 2ng/mL of TGF β (**Figure 14**). Smad 3 activation was observed at about ten minutes over the same time course. These results suggest that, during EMT, SLK most likely plays a scaffolding role that is independent of its kinase activity.

4.7 Kinase-dead SLK does not affect Snail1 upregulation

The absence of SLK activation by TGF β treatment suggests that SLK plays a scaffolding role in EMT. To test this we expressed a kinase-dead version of SLK that contains a point mutation in the ATP-binding site at lysine 63 (Sabourin, Tamai, Seale, Wagner, & Rudnicki, 2000). The endogenous SLK will dimerize with K63R following transfection, resulting in a kinase-inactive complex. This SLK mutant was previously shown to inhibit fibroblast cell migration (Wagner et al., 2008), cell proliferation (O'Reilly et al., 2002) and myoblast fusion (Storbeck et al., 2004). Transfection of K63R into NMuMG cells abrogated total SLK kinase activity (**Figure 15A**).

Supporting a kinase activity-independent role for SLK in EMT, our results demonstrate that cells transfected with K63R can induce Snail1 expression that is indistinguishable from control cells (**Figure 15B**). Taken together, we conclude that SLK kinase activity is not required for TGF β -induced EMT. Therefore, it is more likely that SLK plays a scaffolding role that affects upregulation of Snail1 and its transcriptional targets. To say that SLK is a scaffolding protein is a very broad statement. This could signify that SLK is responsible for assembling key complexes that affect Snail1 stability. One of the key pathways that cross talks with other EMT systems is the Wnt pathway involving GSK3 β and β -Catenin. It has been demonstrated that GSK3- β regulates β -catenin stabilization (He, Saint-Jeannet, Woodgett, Varmus, & David, 1995) (Pierce & Kimelman, 1995) (Dominguez, Itoh, & Sokol, 1995) (Peifer, Pai, & Casey, 1994) (Yost, Torres, Miller, Huang, Kimelman, & Moon, 1996), which in turn regulates gene expression by associating with TCF/LEF1 to increase expression of key mesenchymal markers, including Snail1. One possibility is that SLK scaffolds the interaction of GSK3- β and β -catenin, thus affecting Snail1 upregulation. Alternatively it could regulate the Smad-independent pathways downstream of the TGF β receptors that function in conjunction with the Smad proteins at the level of gene transcription. Some candidate pathways include PI3K/Akt, Ras/Erk/p38 MAPK, and Rho-like GTPases. We can test the involvement of SLK within these pathways using small molecule inhibitors, or siRNA targeted to different proteins in the pathway.

4.8 Future Directions

To further dissect the role of SLK, it is now imperative to map the domain of SLK that is required for the EMT response. Using constructs that we have on hand, we will be in a position

to identify which domain is involved TGF β -induced EMT using Snail1 induction and the cytoskeletal changes as a readout.

The experiments performed in our analysis were done solely based on adenovirus knockdown in cells. To provide a stronger argument, it would be important to use alternate methods of SLK knockdown and demonstrate the same effect. The most effective method involves a genetic deletion of SLK. In this system, we would be able to evaluate long-term effects of SLK deletion on TGF β -induced EMT. This data would allow us to look at E-cadherin downregulation which can take up to 8 days in some cases. We have only been able to demonstrate the short-term effects of SLK deletion, up to 48 hours of TGF β stimulation. It would be important to investigate if SLK deletion would enable a complete block in E-Cadherin downregulation, or if protein levels would eventually decrease over time.

Another important aspect of this analysis is the lack of physiological relevance to EMT signaling in the context of breast cancer. Our lab has already demonstrated that SLK activity responds to heregulin stimulation and HER2 signaling, a key pathway that is upregulated in many breast cancers (Roovers, et al., 2009). Our experiments included analysis in a normal murine mammary gland cell line, NMuMGs. It is important to analyze the effects of SLK downregulation in breast cancer cell lines to support the idea that these effects can be demonstrated in breast cancer. Potential cell lines of interest in these experiments include Py2T cells, which have been shown to respond well to TGF β stimulation both *in vitro* and *in vivo* (Waldmeier, Meyer-Schaller, Diepenbruck, & Christofori, 2012). Including analysis using different cancer cell lines will help support our data demonstrating a key role for SLK in EMT.

We have begun to analyze the role of SLK in other non-canonical pathways by performing mass spectroscopy. In this experiment, we performed an immunoprecipitation using

an antibody against SLK, and treated the samples with TGF β for 30 minutes. Our data demonstrated only one protein that was pulled down with SLK called Annexin 2A. This protein has been shown to bind to STAT3, resulting in its translocation into the nucleus which leads to the upregulation of target genes (Lo, et al., 2007). One paper demonstrates that Annexin 2A is linked to EMT in breast cancer cells, which would validate a role for SLK in this pathway (Wang, et al., 2015). Further investigation is required to manipulate this pathway and elucidate a potential signaling mechanism. We proposed a potential model demonstrating where SLK might fit alongside other non-canonical signaling pathways (**Figure 6**).

4.9 Physiological Relevance

It is our hope that this data can provide new insights into the role of SLK in TGF β -induced EMT in the context of cancer. Perhaps selective SLK inhibition in mammary gland tissues would be an effective way to prevent EMT by reducing Snail1 induction, maintaining E-Cadherin expression, and preventing cell migration and invasion. Current research in our lab is looking into developing selective SLK inhibitors that would disrupt SLK kinase activity. Unfortunately, it appears that SLK kinase activity does not respond to TGF β stimulation *in vitro*, so a focus on protein interaction might be a better option.

Current research is developing reagents to inhibit TGF β signaling pathways. One research group believes that they have identified a small molecule inhibitor that inhibits signaling through Hippo, Wnt, and TGF β signaling pathways. This inhibitor, called C19, was shown to inhibit cell migration and proliferation, and demonstrated anti-tumor activity *in vivo* by inhibiting two key proteins, AMPK and Mst/Lats. The result of this inhibition is the degradation of a key transcription factor, Taz, by GSK3- β (Basu, Lettan, Damodaran, Strellec, Reyes-

Mugica, & Rebbaa, 2014). Taz has been shown to work in conjunction with the β -catenin/TCF/LEF1 and Smad protein complexes in gene transcription downstream of Wnt and TGF β signaling processes (Attisano & Wrana, 2013).. This research presents one potential way to decrease cancer progression.

Other studies have identified methods of preventing TGF β signaling that include the use of neutralizing antibodies, ligand traps, antisense oligonucleotides, and small molecule inhibitors (Korpala & Kang, 2010) (Lampropoulos, Zizi-Sermpetzoglou, Rizos, Kostakis, Nikiteas, & Papavassiliou, 2012) (Connolly, Freimuth, & Akhurst, 2012). There have been multiple studies using neutralizing antibodies that are effective against all three isoforms of TGF β . The first antibody, 2G7, was shown to repress intraabdominal tumor formation and lung metastases in breast cancer cells (Arteaga, Hurd, Winnier, Johnson, Fendly, & Forbes, 1993) and was created by the company Genentech based out of the United States. Another antibody called 1D11, created by Genzyme (Massachusetts, USA) has been shown to reduce invasion of intracranial gliomas (Hulper, et al., 2011). Other studies using the 1D11 antibody have shown a decrease in metastasis using a breast cancer mouse model (Nam, et al., 2008).

Another approach that involves blocking TGF β signaling involved the synthesis of a chimeric protein by Biogen (USA) that contains the extracellular domain of the TGF β RII attached to the Fc portion of a murine IgG heavy chain antibody, referred to as T β RII:Fc. This protein has been shown to prevent breast cancer metastasis in transgenic mice (Yang, et al., 2002). Other studies using this soluble oligonucleotide have shown that it can prevent hepatic fibrosis in rats (George, Roulot, Koteliensky, & Bissell, 1999), and has demonstrated a decrease in cell motility, intravasation, and lung metastases in breast cancer mouse models (Muraoka, et al., 2002), and suppresses pancreatic cancer cell metastasis (Rowland-Goldsmith, et al., 2002).

Other effective methods that are being investigated as clinical treatments employ the use of small molecular inhibitors that block the T β RI from initiating the Smad-dependent signaling cascade. SB431542 was developed by GlaxoSmithKline, and has been shown to selectively inhibit the TGF β receptor I, but shows no effect on the ERK, JNK, or p38 MAP kinase signaling pathways (Inman, et al., 2002). Another small molecule inhibitor called Ki26894, developed by Kirin Brewery Company (Japan), demonstrated decreased bone metastasis in a human breast cancer cell line (Ehata, et al., 2007) (As reviewed in (Syed, 2016)).

In summary, there are multiple methods that are being investigated within the TGF β -dependent signaling pathway in an effort to prevent cell migration and invasion of cancer cells. It is unlikely that there will be one single cancer therapy that proves effective. Studies are moving towards combination therapies that can target multiple areas of cancer progression. It is believed that combined targeted therapies prove more effective than a single-drug approach (as reviewed in (Komarova & Boland, 2013))

5.0 CONCLUSION

We believe that we are on track to identify a novel role for SLK in TGF β -induced EMT. Using a normal murine mammary gland cell line, we studied the effects of SLK-knockdown using a short hairpin adenovirus to evaluate the role of SLK in EMT. Our results demonstrate that SLK depletion significantly impairs TGF β -induced cellular migration and invasion. We also demonstrated using immunofluorescence, that the downregulation and reorganization of epithelial markers is inhibited. Using western blot analysis and immunofluorescence, we showed that Smad3 phosphorylation and localization are not affected in the absence of SLK. In addition, Snail1, fibronectin, vimentin, and E-Cadherin levels are affected in the absence of SLK. Finally, we were able to show that SLK kinase activity is not required for its role in TGF β -induced EMT signaling. Together, these results suggest a novel role for SLK in TGF β -induced epithelial-to-mesenchymal transition and provide novel insights into kinase activity independent roles for this kinase. The isolation of SLK binding proteins and signal transducers will allow the identification and potential inhibition of these pathways to suppress EMT. Further research is required to determine a specific role for SLK in TGF β -induced epithelial-to-mesenchymal transition.

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