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ABSTRACT

Human MAP3K4 (MTK1) functions upstream of mitogen activated protein kinases (MAPKs). In the studies presented herein, MTK1 is shown to be required for human epidermal growth factor receptor 2/3 (HER2/HER3)-heregulin beta1 (HRG) induced extracellular acidification and cell migration in MCF-7 breast cancer cells. Furthermore, it was shown that HRG stimulation leads to association of MTK1 with tyrosine phosphorylated HER3 in MCF-7 and T-47D breast cancer cells. The MTK1/HER3 association was dependent on HER2 activation and was decreased by pre-treatment with the HER2 inhibitor, lapatinib. Furthermore, HER2 does not directly associate with MTK1, but phosphorylates HER3 transiently. MTK1 also has a role in the ERK1/2 MAPK signaling pathway in response to heregulin (HRG) stimulation in T-47D and MCF-7 breast cancer cells. In addition to MTK1, Shc, Grb2 and GIT1 proteins are all involved in the ERK1/2 MAPK pathway in response to growth factor stimulation. MTK1 was also shown to associate with activated ERK1/2, GIT1, Shc, Grb2 and p85 of PI3K in response to heregulin stimulation. ERK1/2 kinase activity is involved in aberrant signaling that leads breast cancer progression. GIT1 is a scaffolding protein that is linked to growth factor mediated ERK1/2 signaling in cell migration. Moreover, we also identify the actin interacting region (AIR) on MTK1 and disruption of actin cytoskeletal polymerization with cytochalasin D inhibited the interaction between HER3 and MTK1, indicating that f-actin (which is needed for cell migration) is required for the MTK1/HER3 association. Additionally, HRG stimulation leads to extracellular
acidification that is independent of cellular proliferation. HRG induced extracellular acidification is significantly inhibited when MTK1 is knocked down in MCF-7 cells. Similarly, pre-treatment with lapatinib significantly decreased HRG induced extracellular acidification. Extracellular acidification is linked with cancer cell migration. We performed scratch assays that show HRG induced cell migration in MCF-7 cells. Knockdown of MTK1 significantly inhibited HRG induced cell migration. Furthermore, pre-treatment with lapatinib also significantly decreased cell migration. Cell migration is required for cancer cell metastasis, which is the major cause of cancer patient mortality. We identify MTK1 in the HER2/HER3-HRG mediated extracellular acidification and cell migration pathway in breast cancer cells.
CHAPTER 1

INTRODUCTION

1.1 MITOGEN ACTIVATED PROTEIN KINASES (MAPKS)

Mitogen activated protein kinases (MAPKs) are activated by a cascade of sequential phosphorylation reactions in response to numerous extracellular stimuli. MEKKs (MAP3Ks) phosphorylate MEKs (MAP2Ks) and MEKs phosphorylate MAPKs (Lange-Carter et al., 1993). One such MAPK is the ERK1/2 (extracellular signal-regulated kinase 1/2). The activation of ERK1/2 is well characterized and required for normal cellular processes, such as proliferation, differentiation and migration (Keshet and Seger, 2010; Roberts and Der, 2007). However, growth factors such as heregulin (HRG) and receptor tyrosine kinase (RTK) proteins that regulate ERK1/2 are often over-expressed in breast cancer cells (Kraus et al., 1989; Lemmon, 2003; Muller et al., 1988; Yarden and Sliwkowski, 2001), leading to unregulated ERK1/2 activity.

This dissertation will focus on the MEKK family member, MEKK4, which is characterized as a MAP3K and named MTK1 (MEKK4 or MAP3K4). Unlike ERK1/2, MTK1 regulation has not been well characterized, even though downstream substrates have been identified (Abell et al., 2007; Mita et al., 2002; Takekawa et al., 1997;
The studies in this dissertation characterize a previously undefined functional role for MTK1 in response to growth factor stimulation of human epithelial breast cancer cells.

The following sections discuss the relevant history of MTK1 as well as new discoveries of several proteins interacting with MTK1 in response to growth factor stimulation. These novel findings shed new light on the functional significance of MTK1 in HER (human epidermal growth factor receptor) signaling in breast cancer. The presentation of this information should shed light on the progression of these studies, while at the same time help to further understand the functional role of MTK1 in breast cancer.

1.1 MTK1 CHARACTERIZATION

MTK1 (MAP Three Kinase 1), also known as MEKK4, is characterized as a MAP3K (Mitogen Activated Protein Kinase Kinase Kinase). It has a molecular weight of approximately 180 kDa and has two isoforms, α and β. MTK1 was cloned from human cDNA and MEKK4 was cloned from mouse cDNA (Gerwins et al., 1997; Takekawa et al., 1998). The human and mouse homologs were independently identified at approximately the same time which resulted in two different names. MTK1 is characterized as a serine/threonine kinase with a carboxyl terminal kinase domain. There are approximately 500 known kinases in the human genome and they represent about 2%
of identified genes (Manning et al., 2002). Kinases modify other proteins (substrates) by transferring the terminal gamma phosphate of ATP (adenosine triphosphate) to the substrate. They are generally regulated by extracellular stimuli and are phosphorylated by other kinases or through auto-phosphorylation. For example, RTKs (receptor tyrosine kinases) are activated by binding of ligand to the extracellular domain of a monomer receptor. The monomer receptor can then auto-phosphorylate itself, which leads to homo or hetero-dimerization with another monomer RTK. Once dimerization is formed, the monomer receptors can trans-phosphorylate each other leading to full activation of the receptors. When fully activated, RTK’s can phosphorylate substrates leading to regulation of intracellular signaling pathways.

MTK1 was originally cloned in 1997 by functional complementation of the osmo-sensitivity of the yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*). The Raf-MEK-ERK MAPK mammalian pathway is regulated by mitogenic stimuli, while the yeast MAPK cascade is regulated by mating pheromones or through environmental stressors i.e., ultraviolet light, osmotic stress, wound stress and cytokine expression (Takekawa et al., 1997). The MEKK family of MAP3Ks was cloned based on the homology to the catalytic domain of the yeast MAP3K mating pathway intermediate protein Ste11 (Sterile 11) (Lange-Carter et al., 1993). When functional Ste11 is absent, the yeast is incapable of mating hence the yeast is sterile. Comparing the sequence homology of human MTK1 and mouse MEKK4 revealed they have 88% amino acid identity and 92% amino acid homology. When Ssk2 (Suppressor of sensor kinase) was cloned from yeast (Maeda et
al., 1995) it became apparent that the MTK1 and MEKK4 amino acid sequences are more homologous to yeast Ssk2 than Ste11 (Bettinger and Amberg, 2007).

Ssk2 is regulated by osmotic stress and functions as a MAP3K (Takekawa et al., 1997). In yeast, extracellular hyperosmolarity is detected by the osmosensor Sln1, which transduces the signal to Ssk2 MAP3K protein (Takekawa et al., 1997). When Ssk2 becomes phosphorylated, it then phosphorylates Pbs2 MAP2K. Pbs2 then phosphorylates and activates HOG1 (High Osmolarity Glycerol response 1) MAPK. HOG1 activation is required for yeast to survive in a high osmolarity environment (Boguslawski, 1992). The high homology between Ssk2, MTK1 and MEKK4 suggested that MTK1 and MEKK4 are regulated by osmotic stress (Figure 1). In fact MEKK4 rescues the loss of Ssk2 resulting in HOG1 MAPK activation, in yeast lacking Ssk2, indicating that MEKK4 compliments Ssk2 in yeast (Takekawa et al., 1997).
FIGURE 1 YEAST AND MAMMALIAN MAPK CASCADES
1.1.2 MAPK REGULATION

The process of signal transduction from extracellular stimuli will often lead to a cascade of phosphorylation reactions. The final phosphorylated kinase in a cascade will translocate to the nucleus and phosphorylate a substrate. These substrates can then associate with DNA, via DNA binding domains, and regulate transcription of mRNA required for protein synthesis. For example, growth factor stimulation results in activation of the Raf, MEK, ERK kinase cascade (Roberts and Der, 2007; Rozakis-Adcock et al., 1992). Raf is a MAP3K and it phosphorylates and activates MEK, which is a MAP2K (Mitogen Activated Protein Kinase, Kinase). MEK then phosphorylates and activates ERK, which is a MAPK (Mitogen Activated Protein Kinase).

In mammalian cells ERK1/2 is regulated by mitogen stimulation and activated by MEK1/2, while p38 and JNK (c-Jun N-terminal Kinase) MAPKs are regulated by environmental stresses like ultraviolet light, would stress, osmotic stress and inflammatory factors (Takekawa et al., 1997). The p38 and JNK MAPK proteins are downstream of MKK3/MKK6 and MKK4/MKK7, respectively. Transfection of mammalian cells with epitope tagged MTK1 or MEKK4 and one potential downstream MAP2K (either, MEK, MKK3, MKK6 or MKK4) followed by immunoprecipitation and \textit{in vitro} kinase assays revealed MTK1 activates both p38 and JNK pathways (Gerwins et al., 1997; Takekawa et al., 1997). In contrast, the MEK1 MAP2K was not activated
indicating that MTK1 and MEKK4 do not regulate the ERK MAPK pathway. Taken together, these results identified MTK1 as a MAP3K that regulates mammalian environmental stress pathways.

1.1.3 MEKK4 REGULATION VIA SIGNAL TRANSDUCTION

Even though MTK1 and MEKK4 regulate the p38 and JNK kinases, the extracellular stimuli required for MTK1 and MEKK4 activation have not been identified. Derbyshire et al. used recombinant MEKK4 as bait to identify proteins interacting with MEKK4. The bait experiment revealed that annexin II associated with MEKK4 (Derbyshire et al., 2005). Annexin II was originally identified as a substrate for the non-receptor tyrosine kinase cSrc (Glenney, 1985). The phosphorylation of annexin II was significantly enhanced with calcium and phosphatidylserine (Glenney, 1985), linking annexin II phosphorylation with calcium signaling and the plasma membrane. Additional experiments by Derbyshire et al. using Far Western blot analysis revealed that the Pyk2 tyrosine kinase associated with MEKK4 (Derbyshire et al., 2005).

Angiotensin II (Ang II) is well documented for regulating cSrc and Pyk2 kinase activity (Berk and Corson, 1997; Murasawa et al., 1998; Schieffer et al., 1996; Tang et al., 2000). AT₁ (Ang II receptor) is a GPCR (G-protein coupled receptor) that regulates the renin-angiotensin system and is strongly correlated to hypertension and cardiovascular diseases
When Ang II binds the extracellular domain of AT$_1$, signal transduction occurs across the plasma membrane into the cell leading to intracellular signaling, i.e. calcium signaling (Startchik et al., 2002). Ang II stimulation of rat aortic vascular smooth muscle cells further revealed the MEKK4/annexin II complex to be dependent on calcium signaling (Derbyshire et al., 2005). Furthermore, an angiotensin II dependent MEKK4/Pyk2 association was observed in rat aortic vascular smooth muscle cells.

Since annexin II was a substrate for cSrc, and Pyk2 associated with MEKK4 in addition to annexin II, both Pyk2 and cSrc tyrosine kinases were potential candidates for regulating MEKK4 activity. Baculovirus infection of Sf9 insect cells with MEKK4 alone or in combination with Pyk2 or vSrc (the constitutively active form of cSrc), followed by MEKK4 immunoprecipitation and phosphotyrosine immunoblots demonstrated that the Pyk2 tyrosine kinase mediated MEKK4 tyrosine phosphorylation (Derbyshire et al., 2005). Furthermore, Pyk2 tyrosine phosphorylation of MEKK4 was shown to regulate MEKK4 kinase activity towards MKK6. Wild type MKK6 and KM MKK6 (kinase inactive) proteins expressed and purified from bacteria were used in kinase assays as substrates for MEKK4. HEK-293 cells expressing AT$_1$ receptor were used to over-express wild-type MEKK4 alone or in combination with Pyk2. Wild type MEKK4 kinase activity towards MKK6 was significantly increased when over-expressed and independent of Ang II stimulation. However, co-expression of MEKK4 and Pyk2 brought MEKK4 kinase activity, toward MKK6, back down to levels consistent with Ang
II stimulation. Additionally, kinase inactive MEKK4 failed to phosphorylate and activate M KK6 (Derbyshire et al., 2005). These data demonstrate that MEKK4 is regulated by activation of a GPCR (Ang II activation of AT1) and subsequent signal transduction in rat aortic vascular smooth muscle cells. Furthermore, MEKK4 kinase activity toward M KK6 is regulated by Pyk2 kinase that leads to a calcium dependent tyrosine phosphorylation of MEKK4.

Halfter et al. utilized the HaCaT human keratinocyte cell line to further characterize the calcium dependent interaction of MEKK4 with annexin II and Pyk2 using IFNγ (Interferon-γ). IFNγ is a cytokine involved in antiviral protection, immune-regulation and growth inhibition (Bach et al., 1997). Additionally, IFNγ has been shown to elicit calcium flux in several types of human cells including neutrophils, lymphocytes, and microglia (Aas et al., 1999; Buntinx et al., 2002; Franciosi et al., 2002), linking IFNγ to calcium signaling. The IFNGR (IFNγ receptor) has two different subunits, IFNGR-α and IFNGR-β which form dimers when IFNγ binds the IFNGR-α subunit. Each subunit has intracellular cytoplasmic binding sites for JAK (Janus kinase) proteins JAK-1 and JAK-2. The IFNGR-α has a JAK-1 binding site and IFNGR-β has a JAK-2 binding site. Like Ang II, IFNγ regulates signal transduction across the plasma membrane into the cell. Upon IFNγ binding to the extracellular domain of IFNGR, hetero-dimerization of these receptors occurs, followed by JAK-1 and JAK-2 tyrosine phosphorylation of IFNGR intracellular domains, which lead to recruitment of STAT (signal transducer and activator
of transcription). STAT proteins are then tyrosine phosphorylated by JAKs, form STAT dimers and localize to the nucleus to regulate transcription (Bach et al., 1997).

Stimulation of HaCaT cells with IFNγ leads to the tyrosine phosphorylation of MEKK4. Extracellular calcium chelation using EGTA had no effect on IFNγ dependent MEKK4 tyrosine phosphorylation. However, intracellular calcium chelation using BAPTA/AM [bis-(o-aminophenoxy) ethane-N,N',N',N'-tetra-acetic acid tetrakis (acetoxy methyl ester)] followed by IFNγ stimulation inhibited MEKK4 tyrosine phosphorylation. Furthermore, intracellular calcium chelation inhibited IFNγ dependent annexin II and Pyk2 association with MEKK4 indicating intracellular calcium signaling is required for MEKK4/annexin II/Pyk2 association (Halfter et al., 2005). Additionally, IFNγ mediated tyrosine phosphorylation of MEKK4 regulates kinase activity towards MKK6. MEKK4 immunoprecipitated from IFNγ stimulated HaCaT cells was used to perform in vitro kinase assays with purified wild type MKK6 or KM MKK6 as a substrate. IFNγ stimulation resulted in activation of MEKK4 kinase activity towards MKK6. These experiments revealed MEKK4 is regulated by cytokine receptor (IFNγ activation of INFGR) activation and subsequent signal transduction in HaCaT cells, leading to a Pyk2 dependent tyrosine phosphorylation of MEKK4 and subsequent regulation of MKK6 activation. Furthermore, there was a calcium dependent association of MEKK4 with Pyk2 and annexin II in HaCaT cells (Halfter et al., 2005). Taken together, these data revealed MEKK4 was regulated by calcium signaling in both rat aortic vascular smooth muscle cells and human keratinocytes.
1.1.4 MTK1 CATALYTIC ACTIVITY

The heart is one of the first organs to develop and congenital malformations occur at a rate of about one in one hundred (Hoffman and Kaplan, 2002). Mutation of lysine in the active site of MEKK4 produces a kinase inactive protein. Kinase inactive (KM) MEKK4 attenuates developmental epithelial to mesenchymal transformation in mouse atrioventricular canal and ventricular heart explants (Stevens et al., 2006). A knock-in mutation of kinase-inactive KM MEKK4 was introduced in mice and the pups die at birth from skeletal malformations and neural tube defects (Abell et al., 2005). These findings emphasize the importance of MEKK4 kinase activity during development. In addition to kinase activity, MEKK4 protein expression is also important in development. MEKK4 is highly expressed in the developing neuroepithelium and MEKK4 knockout mice display neural tube defects resulting in exencephaly and spina bifida (Chi et al., 2005). MEKK4 knockout mice also display a congenital malformation of the cerebral cortex and MEKK4 RNA interference impairs neuronal cell migration during brain development (Sarkisian et al., 2006).

MTK1 catalytic activity is activated by binding of GADD45 (Growth Arrest and DNA Damage-inducible 45) to the amino-terminal domain of MTK1 (Takekawa and Saito, 1998). When the amino- and carboxyl-terminal domains of MTK1 associate, this
interaction is auto-inhibitory, blocking kinase activity. GADD45 association with MTK1 causes dissociation of the MTK1 amino-terminal and carboxyl-terminal domains leading to dimerization, auto-phosphorylation and activation of MTK1 (Miyake et al., 2007).

GADD45 proteins are correlated with cell cycle control, DNA repair and genotoxic stress. There are three characterized isoforms of GADD45, which include GADD45α, GADD45β and GADD45γ. They are small acidic proteins that facilitate protein-protein interactions. Additionally, GADD45 proteins are pro-apoptotic making them prime candidates for inhibiting oncogenesis (Tamura et al., 2012). GADD45β has been linked to autophagy that is mediated through the MEKK4 and p38 MAPK pathway (Keil et al., 2013). Autophagy is a process where dysfunctional or unrequired cell components are degraded within the cell by lysosomal machinery in times of cellular stress or starvation. Furthermore, over-expression of GADD45β and MEKK4 in NIH/3T3 fibroblasts cells induced phosphorylation of p38, while co-expression of GADD45β and dominant negative (kinase inactive) MEKK4 did not activate p38 (Keil et al., 2013). The active p38 localized to autophagosomes and was shown to inhibit autophagy, linking GADD45β, MEKK4 and p38 to autophagy (Keil et al., 2013).
1.2 RTK REGULATION OF MTK1

The following sections provide background information regarding the proteins that were characterized in this dissertation as associating with MTK1 in response to growth factor stimulation in human breast cancer cells. Additionally, previously unknown growth factor dependent functional roles of MTK1 identified in this dissertation will be introduced. The mouse homolog of MTK1, MEKK4 is regulated by GPCR (AT$_1$ receptor) and cytokine receptor (INFGR) activation and subsequent signal transduction pathways. These transduced signaling events lead to MEKK4 tyrosine phosphorylation and subsequent MEKK4 catalytic activity toward MKK6 (Derbyshire et al., 2005; Halfter et al., 2005). The tyrosine phosphorylation of MEKK4 was mediated through a non-receptor tyrosine kinase, Pyk2. Activation of RTKs (Receptor Tyrosine Kinases) via growth factor stimulation initiates signal transduction similar to GPCR and cytokine receptors. However, RTKs can directly phosphorylate substrates on tyrosine residues when activated by growth factor stimulation. The focus of this dissertation was to determine whether MTK1 was regulated by RTK in response to growth factor stimulation.
1.2.1 RTK (RECEPTOR TYROSINE KINASE)

Mammalian regulated cell growth and differentiation relies heavily on the extracellular binding of growth factors to their cell surface receptors (Iwashita and Kobayashi, 1992). This form of signal transduction leads to the activation of second messenger signaling that regulate intracellular signaling events to create cellular homeostasis (Iwashita and Kobayashi, 1992). The insulin receptor (IR) is a prime example of RTK activation that leads to second messenger signaling. The IR is composed of two identical α-subunits and two identical β-subunits that are linked together via disulfide bonds (Ellis et al., 1991; Youngren, 2007). The extracellular α-subunits have insulin binding sites and the transmembrane spanning intracellular β-subunits have catalytic activity. When insulin binds to the α-subunits, this causes a conformational change of the β-subunits triggering autophosphorylation of tyrosine residues and subsequent catalytic activity of the β-subunits (Youngren, 2007). Tyrosine phosphorylation of the IR leads to the association IR substrates.

Two examples of IR substrates that associate with activated IR are the Insulin Receptor Substrate (IRS) and Src Homology and Collagen (Shc) proteins. Both IRS and Shc have PTB (protein tyrosine binding) domains (Pelicci et al., 1996; Wolf et al., 1995) that compete for association with intracellular tyrosine phosphorylated NPXY motifs of the IR. Once association occurs, both Shc and IRS are tyrosine phosphorylated by IR and act
as docking sites for other signaling proteins within the cell (Youngren, 2007). The Shc pathway of IR second messenger signaling leads to the activation of the Ras, Raf, MEK, ERK MAPK kinase cascade (Egan et al., 1993; Kumar et al., 1995; Rozakis-Adcock et al., 1992; Skolnik et al., 1993). The IRS branch, of IR second messenger signaling, leads to the recruitment of phosphatidylinositol 3-kinase (PI3K) and subsequent activation of AKT (Youngren, 2007). Recruitment and activation of these second messenger signaling events leads to cellular growth, protein synthesis and ultimately translocation of glucose transporter to the plasma membrane facilitating cellular glucose uptake (Youngren, 2007).

Another example of RTK activation occurs by binding of ligand to the extracellular domain of a monomer receptor. The monomer receptor can then auto-phosphorylate itself, via cytoplasmic intracellular domain catalytic activity, which leads to homo or hetero-dimerization with another RTK. Once dimerization is formed, the receptors can trans-phosphorylate each other leading to full activation of the receptors. When fully activated, RTK’s can phosphorylate substrates leading to regulation of intracellular signaling pathways.

A well characterized RTK pathway that is activated through receptor dimer formation is the epidermal growth factor receptor (EGFR), also known as human epidermal growth factor receptor 1 (HER1), signal transduction pathway. The EGFR is required for normal
human development and growth, however over-expression of EGFR or its ligand epidermal growth factor (EGF) can result in constitutive activation of the receptor, leading to initiation and propagation of many types of cancer (Bartolotti et al., 2012; Khazaie et al., 1993; Lemmon, 2003).

### 1.2.2 RTK HER1-4 (Human Epidermal Growth Factor Receptor1-4)

Receptor tyrosine kinases (RTK’s) and the growth factors that regulate them, such as heregulin (HRG) are often over-expressed in breast cancer cells (Kraus et al., 1989; Lemmon, 2003; Muller et al., 1988; Yarden and Sliwkowski, 2001). HER1-4 proteins are a class of RTK’s that are required for cell proliferation and differentiation during development (Darcy et al., 2000; Eccles, 2011). HER2 is an orphan receptor with no known ligand. HER2 can form a heterodimer with EGFR (HER1), HER3 or HER4 and is often over-expressed in breast cancer (Menard et al., 2000). HER4 expression correlates with favorable prognosis, while EGFR, HER2 and HER3 correlate with poor prognosis in breast cancer patients (Fuchs et al., 2006). The growth factor, HRG, is a ligand for HER3 and HER4, however HER3 is not kinase active and requires heterodimerization with either EGFR, HER2 or HER4 for activity (Carraway et al., 1994; Lyne et al., 1997; Stern, 2008). Furthermore, HER2/HER3 is the preferred heterodimer for
HRG and produces strong mitogenic signaling that is linked to cancer (Alroy and Yarden, 1997; Amin et al., 2012; Stern, 2008; Way and Lin, 2005).

HER3 is unique among the HER family of receptors in that it is not catalytically active and therefore requires heterodimerization with another RTK in order to become tyrosine phosphorylated. Additionally, HER3 has six consensus binding sites at its carboxyl terminus for p85 of PI3K (Hellyer et al., 1998). When tyrosine phosphorylated, these six carboxyl terminus YXXM motifs have been shown to specifically associate with the Src homology (SH2) domain of p85 (Hellyer et al., 1998). Furthermore, HER3 has a proline rich domain that forms a consensus binding site for the SH3 domain of p85 (Hellyer et al., 1998). In these studies, tyrosine 1289 phosphorylated (an YXXM motif) HER3 was identified as associating with MTK1 upon HRG stimulation of human epithelial breast cells. Additionally, HER2 catalytic activity was identified as being required for HRG dependent formation of the MTK1/HER3 heterodimer. However, direct association of HER2 with the MTK1/HER3 complex was not observed, suggesting the HRG dependent HER2 tyrosine phosphorylation of HER3 occurs transiently.

1.2.3 BREAST CANCER CELL MODELS

Cell lines used in these studies include T-47D, MCF-7, and MDA-MB-231 human epithelial breast cancer cells, HEK 293 human embryonic kidney cells and Sf9 insect cells. MCF-7 and T-47D cells express moderate levels of the HER1-4 proteins (Beerli
and Hynes, 1996; Carraway et al., 1994). MDA-MB-231 cells are a triple negative cell line, which do not express estrogen receptor, progesterone receptor or HER2, but do express HER3 (Teixido et al., 2012). The HER protein expression profiles of these breast cancer cell lines made them ideal for studying growth factor dependent MTK1 and HER1-4 signaling. HEK 293 cells were utilized because they are easily transfected. Sf9 insect cells were utilized to produce recombinant MEKK4 proteins used in biochemical assays.

1.2.4 SHC (SRC HOMOLOGY AND COLLAGEN) PROTEINS

Shc proteins regulate ERK1/2 activity. In this dissertation, Shc was identified as constitutively associating with MTK1 in human epithelial breast cancer cells. MTK1/Shc association increased significantly after HRG stimulation and Shc was highly tyrosine phosphorylated. There are three characterized Shc protein isoforms, consisting of 46, 52 and 66 kDa. Shc is tyrosine phosphorylated by activated RTKs in response to growth factor stimulation (Basu et al., 1994; Rozakis-Adcock et al., 1992). Once tyrosine phosphorylated, Shc forms a complex with the adapter protein Grb2 (Growth factor receptor-bound 2), which leads to the activation of SOS (son of sevenless) and the Ras signaling pathway, resulting in activation of ERK1/2 (Egan et al., 1993; Kumar et al., 1995; Rozakis-Adcock et al., 1992; Skolnik et al., 1993). The Shc proteins have three primary domains, an amino-terminal PTB (protein tyrosine binding) domain that
associates with tyrosine-phosphorylated NPXY motifs, an internal CH1 (collagen homology 1) domain that is tyrosine phosphorylated, and a SH2 (Src homology 2) domain that associates with phosphotyrosine residues (Pelicci et al., 1996). These three domains allow Shc to associate with other proteins under specific post-translational modifications and regulate their activity in various signaling pathways within the cell.

When a RTK becomes activated, via tyrosine phosphorylation, Shc can associate with the receptor either through its PTB or SH2 domain. Shc is tyrosine phosphorylated during different types of growth factor stimulation. For example, EGF (epidermal growth factor) stimulation of the EGFR (epidermal growth factor receptor) leads to the tyrosine phosphorylation of Shc (Basu et al., 1994; Rozakis-Adcock et al., 1992). Once tyrosine phosphorylated, Shc is able to form a complex with the adapter protein Grb2. Tyrosine phosphorylation of Shc is required for association of Grb2 and subsequent activation of the Ras ERK1/2 signaling pathway that leads to proliferation of human breast cancer cells (Clark et al., 1996).

1.2.5 ERK1/2 ACTIVATION

The activation of ERK1/2 is required for normal cellular processes, such as proliferation, differentiation and migration (Keshet and Seger, 2010; Roberts and Der, 2007). There are two isoforms of ERK, ERK1 with molecular weight of 44 kDa and ERK2 with a
molecular weight of 42 kDa and they are known to function similarly (Boulton and Cobb, 1991). Growth factors that regulate ERK1/2 are often over-expressed in breast cancer cells (Kraus et al., 1989; Lemmon, 2003; Muller et al., 1988; Yarden and Sliwkowski, 2001), leading to unregulated ERK1/2 activity. In these studies, activated ERK1/2 was identified to associate with MTK1 in response to HRG stimulation in human breast cancer cells.

As mentioned previously, MAPK proteins are regulated by a cascade of phosphorylation reactions. There are four mammalian MAPK protein cascades that have been characterized in the past twenty years, extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal Kinase (JNK), p38 and ERK5 (Keshet and Seger, 2010). ERK1/2 is regulated after activation of cytokine receptors, G-protein coupled receptors (GPCR) and receptor tyrosine kinases (RTK) (Gerwins et al., 1997; Johnson and Vaillancourt, 1994). Upon growth factor stimulation the serine/threonine kinase, Raf, phosphorylates and activates MEK, then MEK phosphorylates ERK1/2 on tyrosine and threonine residues of the TEY motif resulting in the activation of ERK1/2 MAPK (Anderson et al., 1990; Lange-Carter et al., 1993; Roberts and Der, 2007; Rozakis-Adcock et al., 1992). Once activated, ERK1/2 forms dimers, localizes to the nucleus and regulates gene transcription by activation of transcription factors such as, ELK-1, c-Jun, and c-Fos (Chen et al., 1992; Rao and Reddy, 1993).
1.2.6 GIT1 (G-PROTEIN COUPLED RECEPTOR KINASE INTERACTING PROTEIN 1)

G-protein coupled receptor kinase interacting protein 1 (GIT1) is a scaffolding protein that associates with ERK1/2 in response to growth factors (Yin et al., 2005; Zhang et al., 2010a). In these studies, tyrosine phosphorylated GIT1 was identified as associating with MTK1. GIT1 consists of multiple domains that function as recruitment sites for other proteins. At the amino terminus, GIT1 has an ARF-GTPase activating protein (ARF-GTP) domain followed by three ankyrin (ANK) repeats, an internal Spa2 homology (SHD) domain, a coiled-coiled (CC2) domain and a carboxyl terminal paxillin binding site (PBS) (Hoefen and Berk, 2006). GIT1 acts as a scaffold protein that is required for activation of the MEK1-ERK1/2 pathway in response to angiotensin II (AngII) and EGF stimulation (Yin et al., 2004). Additionally, GIT1 co-localizes with ERK1/2 at focal adhesions and the CC2 domain is required for the GIT1/ERK1/2 association (Zhang et al., 2010). GIT1 is also tyrosine phosphorylated and regulated by the c-Src non-receptor tyrosine kinase in response to agonist stimulation (Hoefen and Berk, 2006; Sato et al., 2008).
1.2.7 PI3K (PHOSPHATIDYLINOSITOL 3-KINASE)

Phosphatidylinositol 3-kinase (PI3K) proteins mediate activation of signaling events required for initiation and progression of cancer. Specifically, the functional outcomes of PI3K activity include cell motility, migration, invasion, angiogenesis, cell survival and autophagy (McAuliffe et al., 2010). The PI3K proteins form heterodimers comprised of a catalytic subunit and a regulatory subunit. There are four characterized catalytic subunits, p110α, p110β, p110γ and p110δ, which catalyze the phosphorylation of phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) (Fruman, 2010). Each catalytic subunit has five characterized functional domains, an amino-terminal p85 binding site, flanked by a Ras binding domain, a C2 domain, a helical domain and a carboxyl-terminal catalytic domain (Fruman, 2010). The catalytic subunit binds to one of five regulatory subunits p85α, p55α, p50α, p85β or p55γ (Fruman, 2010). The p85 regulatory subunits have three SH2 domains; two SH2 domains flank an internal SH2 domain. The flaking SH2 domains associate with phosphotyrosine YXXM motifs. For example, HER3 has several YXXM motifs which, when phosphorylated, function as recruitment sites for the SH2 domain of the p85 regulatory subunit of PI3K (Hellyer et al., 1998). The p85 internal SH2 domain associates with the p110 catalytic subunit via the p110 amino-terminus p85 binding site. Additionally, the p85 regulatory subunit has an amino-terminal SH3 domain, that can associate with proline rich epitopes (Fruman, 2010). In the studies presented here in this dissertation, p85α of PI3K was
identified as associating with MTK1 in response to HRG or insulin stimulation of human breast cancer cells.

PI3K phosphorylation of membrane associated phosphatidylinositol is followed by recruitment of Akt [also known as protein kinase b (PKB)] and phosphoinositide dependent kinase 1 (PDK1) to the plasma membrane (McAuliffe et al., 2010). PDK1 then phosphorylates and activates Akt leading to the activation of downstream signaling pathways that mediate cancer progression, such as cell migration and metastasis (McAuliffe et al., 2010). PI3K signaling is regulated by tumor suppressor phosphatase and tensin homolog deleted from chromosome 10 (PTEN). PTEN regulates PI3K signaling by dephosphorylation of membrane bound phosphatidylinositols (McAuliffe et al., 2010).

1.2.8 FILAMINOTOUS ACTIN

Actin is expressed in all eukaryotic cells and can be present at concentrations as high as 100 µM (Cooper, 1991). Under non-polymerized conditions, globular actin (g-actin) has a molecular weight of 42 kDa (Cooper, 1991). Actin also forms helical polymers called filamentous actin (f-actin) (Cooper, 1991). F-actin is formed by polymerization of long chains of g-actin monomers coupled together. Actin mediates many cellular functions,
including muscle contraction, vesicular trafficking and cell motility and migration (Cooper, 1991; Kee et al., 2009; Mooren et al., 2012; Porat-Shliom et al., 2013).

Cell migration requires actin polymerization and intracellular coordination of actin binding proteins, which are regulated by HER2 and downstream signaling proteins (Feldner and Brandt, 2002). For example, HRG stimulation of breast cancer cells enhances the conversion of g-actin to f-actin increasing cell migration (Adam et al., 1998; Hijazi et al., 2000). Additionally, HER3 is regulated by HRG stimulation through HER2 kinase activity, which links HER3 to actin cytoskeletal reorganization and cell migration.

The yeast homolog of MTK1, Ssk2p, is an example of an actin binding protein. The actin cytoskeleton is disassembled rapidly in response to osmotic stress and reassembles only after osmotic balance has been reestablished (Yuzyuk et al., 2002a). Ssk2p has an actin interacting region (AIR) that is required for actin cytoskeleton recovery after osmotic stress (Yuzyuk et al., 2002a). Alignment of the AIR of Ssk2p with MEKK4 revealed 17/40 amino acids are identical between these proteins and 25/40 are highly conserved, suggesting that MTK1/MEKK4 might interact with actin. In these studies, actin was identified as associating with MTK1/MEKK4. The MTK1/MEKK4 association with actin was identified as being mediated through an AIR located on MTK1/MEKK4. Additionally, f-actin was shown to be required for HRG dependent MTK1/HER3 association in human epithelial breast cancer cells (Sollome et al., 2013).
1.2.9 MTK1, EXTRACELLULAR ACIDIFICATION & CELL MIGRATION

Cancer cells have increased glycolytic metabolism leading to acid loading and as a result, excess protons are excreted from the cell by the up-regulation of proton transporters (Stock et al., 2005). Cell migration is a possible functional outcome to acid excretion because this creates micro acidic extracellular environments that are favorable for cell migration (Stock and Schwab, 2009). In fact, human melanoma cells (A-07, D-12 and T-22) cultured in acidic media had up-regulated expression of proteolytic enzymes MMP2, MMP9, cathepsin B and cathepsin L. Additionally, these cells expressed increased levels of proangiogenic factors such as, vascular endothelial growth factor A (VEGFA) and interleukin 8 (IL8) (Rofstad et al., 2006). Furthermore, extracellular acidification of A-07, D-12 and T-22 melanoma cells enhanced their metastasis and invasive potential (Rofstad et al., 2006). In the studies reported in this dissertation, HRG stimulation of human epithelial breast cancer cells led to extracellular acidification, independent of proliferation, and cell migration. Additionally in these studies MTK1 was shown to be required for HRG dependent extracellular acidification and cell migration.

HER2 over-expression occurs in roughly 20% of all breast cancers and contributes to metastatic potential and poor prognosis in breast cancer patients. The HER2/HER3 heterodimer is a potent oncogenic signaling complex that drives proliferation and migration (Asrani et al., 2013). HER2 over-expression in estrogen positive cells is
associated with tamoxifin drug resistance in breast cancer (Kurokawa and Arteaga, 2003; Kurokawa et al., 2000; Shou et al., 2004). The drugs trastuzumab and lapatinib show high efficacy with HER2 positive patients, however drug resistance still persists (Bedard et al., 2009; Engel and Kaklamani, 2007; Esteva and Pusztai, 2005; Scaltriti et al., 2009; Stern, 2012). HER3 protein expression was shown to be up-regulated with lapatinib treatment, compensating for HER2 inhibition, and HER3 phosphorylation occurred by residual HER2 expression limiting the efficacy of lapatinib treatment (Garrett et al., 2011). Therefore, HER3 over-expression and recovery of phosphorylation appears to be a compensatory mechanism in response to drug targeting of HER2.

Additionally, Vaught et al. showed HER3 to be required for HER2 induced pre-neoplastic changes and tumor formation in breast epithelium. Cre-mediated ablation of HER3 in HER2 expressing mammary specific mouse models revealed HER3 to be required for transformation of the mammary epithelium (Vaught et al., 2012). Furthermore, palpable tumor penetrance decreased from 93.3% to 6.7% when HER3 was ablated (Vaught et al., 2012). HER3 ablation also decrease Akt and ERK1/2 phosphorylation and increased tumor responsiveness to the HER2 kinase inhibitor lapatinib (Vaught et al., 2012). The studies mentioned above clearly reveal HER3 expression as being required for HER2 mediated tumor formation and progression. Moreover, HER3 expression is correlated to patient responsiveness to anti-HER2 pharmaceuticals like lapatinib. These observations have shed new light on HER3 as an attractive new molecular target for treating HER2 positive breast cancer. In the studies
presented in this dissertation, tyrosine phosphorylated HER3 was observed associating with MTK1 in response to HRG stimulation. Even though HER2 kinase activity was shown to be required for MTK1/HER3 association, HER2 was not observed associating with MTK1. This previously uncharacterized novel interaction between MTK1 and HER3 in response to HRG stimulation suggests the possibility of a new heterodimer (MTK1/HER3) and makes MTK1 an attractive molecular target for development of antineoplastic pharmaceuticals for treatment of HER2/HER3 positive breast cancers.

1.2.10 BREAST CANCER IN THE UNITED STATES

According to the CDC (Centers for Disease Control) in 2009 breast cancer was the most commonly diagnosed form of cancer for women in the United States (Figure 2). Risk factors associated with developing breast cancer include but are not limited to age, gender, diet, sedentary life style, extensive use of hormonal replacement therapy, chemical environmental exposures and genetic predisposition (Mitra et al., 2004; Thomson, 2012; Welnicka-Jaskiewicz and Jassem, 2003). Invasive breast cancer causes over 90% of breast cancer related deaths (Wang, 2010). Furthermore, mortality is not generally associated with the primary tumor, but instead is a result of metastasis to other organs (Scully et al., 2012). Therefore, early diagnosis is paramount for a favorable prognosis in breast cancer patients. Once diagnosed, treatment options include surgical
excision of the tumor, radiation therapy, and chemotherapy using antineoplastic pharmaceuticals.
FIGURE 2. TOP 10 CANCERS FOR FEMALES IN THE UNITED STATES.

According to the Centers for Disease Control (CDC) 2009 statistics, breast cancer is number one cancer for women in the United States.
To increase the patient’s chances of survival, oncologists usually recommend all three treatments simultaneously. Early detection can be achieved by routine breast examination; the American Cancer Society recommends that women ages 20 to 30 years old should have a clinical breast examination performed during their periodic physical exam every three years. Furthermore, women over the age of 40 should have a clinical breast exam annually performed by their physician.

1.2.11 BREAST CANCER, TUMOR SUPRESSORS AND ONCOGENES

Like other forms of cancer, breast cancer is initiated by mutations in genes that regulate cellular processes. Tumor suppressors also known as anti-oncogenes protect the cell from the progression to cancer. Oncogenes have the potential to cause cancer when gene mutation and/or gene duplication occur. Simultaneous inactivation of tumor suppressors and up-regulation of oncogene activity lead to breast cancer. One example of a tumor suppressor is the breast cancer type 1 susceptibility protein (BRCA1), which is responsible for repairing damaged DNA. When both BRCA1 alleles are mutated, double stranded DNA breaks are left to non-specific processing leading to random rejoining of DNA which increases the chance of further gene mutations and cancer progression (Friedenson, 2007). Examples of a class of oncogenes are the human epidermal growth factor receptors 1-4 (HER1-4). Even though these receptors are required for normal human development and growth (Darcy et al., 2000; Eccles, 2011), they can potentiate
cancer progression through unregulated activity in response to growth factors. In fact, the HER1, HER2 and HER3 proteins are highly correlated with breast cancer (Stern, 2008; Way and Lin, 2005), which has made these receptors attractive targets for antineoplastic pharmaceutical development in recent years.

The proteins discussed in the preceding sections are identified as interacting with MTK1. The following studies reveal MTK1 to be regulated by HER signaling in response to HRG stimulation in human epithelial breast cancer cells. The characterization of MTK1 associating with these proteins has shed new light on the functional significance of MTK1 in HER2/HER3 positive breast cancer.

The following studies address the hypothesis that MTK1 is regulated by RTK’s in response to HRG stimulation in breast cancer cells. Specifically, MTK1 was identified associating with tyrosine phosphorylated HER3, tyrosine phosphorylated 52 kDa Shc, Grb2, tyrosine phosphorylated GIT1, activated ERK1/2 and p85 of PI3K in response to HRG stimulation. MTK1 was also shown to have a constitutive association with Shc, Grb2, GIT1, ERK1/2 and actin, revealing MTK1 to have scaffolding properties. Additionally, an AIR domain was identified on MTK1 and f-actin was shown to be required for the MTK1/HER3 association. The HRG dependent MTK1/HER3 association was shown to require HER2 kinase activity, however HER2 was not observed associating with MTK1. MTK1 was also shown to be required for HRG mediated
extracellular acidification and cell migration. Identification of these functional roles makes MTK1 an attractive candidate for the development of anti-neoplastic pharmaceuticals for treatment of HER2/HER3 positive breast cancers.
CHAPTER 2

MTK1 SIGNALS THROUGH HER2/HER3 AND HEREGULIN TO REGULATE EXTRACELLULAR ACIDIFICATION AND CELL MIGRATION


This chapter has been published. The text and data are largely the same as the publication, but has been fitted to the style of the dissertation.

2.1 INTRODUCTION

Mitogen activated protein kinases (MAPKs) are regulated by various extracellular stimuli resulting from a cascade of sequential phosphorylations. MAPKs, such as the extracellular signal-regulated kinases (ERKs), are phosphorylated by MEKs and MEKs are phosphorylated by MEKKs (Lange-Carter et al., 1993). The MEKK family of MAP3Ks was cloned based on homology to the catalytic domain of the yeast MAP3K, Ste11 (Lange-Carter et al., 1993). MEKK4 (MAP3K4) was cloned using cDNA isolated from mouse (Gerwins et al., 1997), while MTK1 (MAP3K4) was cloned using human
cDNA (Takekawa et al., 1997) and the sequence homology between the two proteins is 88% amino acid identity and 92% amino acid homology. When Ssk2 was cloned from yeast (Maeda et al., 1995) it became apparent that the MEKK4 and MTK1 amino acid sequences are more homologous to yeast Ssk2p than Stellp (Bettinger and Amberg, 2007). Ssk2p is regulated by osmotic stress (Takekawa et al., 1997). In yeast lacking Ssk2p, MEKK4 rescues the loss of Ssk2p resulting in p38 MAPK activation indicating that MEKK4 compliments Sskp2 in yeast (Takekawa et al., 1997).

The heart is one of the first organs to develop and congenital malformations occur at a rate of about one in one hundred (Hoffman and Kaplan, 2002). Mutation of lysine in the active site of MEKK4 produces a kinase inactive protein. Kinase inactive MEKK4 attenuates developmental epithelial to mesenchymal transformation in mouse atrioventricular canal and ventricular heart explants (Kirk et al., 2006). A knock-in mutation of kinase-inactive MEKK4 was introduced in mice and the pups die at birth from skeletal malformations and neural tube defects (Abell et al., 2005). These findings emphasize the importance of MEKK4 kinase activity during development. In addition to kinase activity, MEKK4 protein expression is also important in development. MEKK4 is highly expressed in the developing neuroepithelium and MEKK4 knockout mice display neural tube defects resulting in exencephaly and spina bifida (Chi et al., 2005). MEKK4 knockout mice also display a congenital malformation of the cerebral cortex and MEKK4 RNA interference impairs neuronal cell migration (Sarkisian et al., 2006).
Human MAP3K4 catalytic activity is activated by binding of GADD45 to the amino-terminal domain of MTK1 (Takekawa and Saito, 1998). In contrast when the amino- and carboxyl-terminal domains of MTKs associate, this interaction is auto-inhibitory, blocking kinase activity. GADD45 association with MTK1 causes dissociation of the MTK1 amino-terminal and carboxyl-terminal domains leading to dimerization, auto-phosphorylation and activation of MTK1 (Miyake et al., 2007). Human MAP3K4 (MTK1) and the mouse homolog (MEKK4) regulate M KK6, which is upstream of stress activated p38 MAPK (Mita et al., 2002; Takekawa et al., 1997; Takekawa and Saito, 1998). In addition, stress induced activation of MEKK4 leads to activation of MEK4/7 and JNK (Abell et al., 2007).

Receptor tyrosine kinases (RTK’s) and the growth factors that regulate them, such as heregulin (HRG) are often over-expressed in breast cancer cells (Kraus et al., 1989; Lemmon, 2003; Muller et al., 1988; Yarden and Sliwkowski, 2001), leading to activation of ERK1/2 activity, cell cycle progression (Fiddes et al., 1998) and cell migration (Krueger et al., 2001; Wu et al., 2008). The human epidermal growth factor receptors (HER) 1-4 are required for cell proliferation and differentiation during development (Darcy et al., 2000; Eccles, 2011). HER2 is an orphan receptor with no known ligand. HER2 can form a heterodimer with EGFR, HER3 or HER4 and is often over-expressed in breast cancer (Menard et al., 2000). HER4 expression correlates with favorable
prognosis, while EGFR, HER2 and HER3 correlate with poor prognosis in breast cancer patients (Fuchs et al., 2006). The growth factor, heregulin, is a ligand for HER3 and HER4, however HER3 is not kinase active and requires hetero-dimerization with either EGFR, HER2 or HER4 for activity (Carraway et al., 1994; Lyne et al., 1997; Stern, 2008). Furthermore, HER2/HER3 is the preferred heterodimer for heregulin and produces strong mitogenic signaling that is linked to cancer (Alroy and Yarden, 1997; Amin et al., 2012; Stern, 2008; Way and Lin, 2005).

HER2 over expression in estrogen positive cells is associated with tamoxifin drug resistance in breast cancer (Kurokawa and Arteaga, 2003; Kurokawa et al., 2000; Shou et al., 2004). The drugs trastuzumab and lapatinib show high efficacy with HER2 positive patients, however drug resistance still persists (Bedard et al., 2009; Engel and Kaklamani, 2007; Esteva and Pusztai, 2005; Scaltriti et al., 2009; Stern, 2012). HER3 protein expression was shown to be up-regulated with lapatinib treatment, compensating for HER2 inhibition, and HER3 phosphorylation occurred by residual HER2 expression limiting the efficacy of lapatinib treatment (Garrett et al., 2011). Therefore, HER3 overexpression and recovery of phosphorylation appears to be a compensatory mechanism in response to drug targeting of HER2. HER3 requires the catalytic activity of other members of the HER family for phosphorylation. A unique feature of HER3 is the six YXXM binding motifs that when phosphorylated function as recruitment sites for the SH2 domain of p85 of phosphoinositide kinase 3 (PI3K) leading to increased cell motility, invasion and metastasis (Hellyer et al., 1998).
Cell migration requires actin polymerization and intracellular coordination of actin binding proteins, which are regulated by HER2 and downstream signaling proteins (Feldner and Brandt, 2002). For example, heregulin stimulation of breast cancer cells enhances the conversion of globular actin (g-actin) to filamentous actin (f-actin) increasing cell migration (Adam et al., 1998; Hijazi et al., 2000). Additionally, HER3 is regulated by HRG stimulation through HER2 kinase activity, which links HER3 to actin cytoskeletal reorganization and cell migration. Ssk2p is an example of an actin binding protein and is a homolog of MTK1. Ssk2p has an actin interacting region (AIR) that is required for actin cytoskeleton recovery after osmotic stress (Yuzyuk et al., 2002b). Despite the evidence for Ssk2p involvement in actin cytoskeletal reorganization, a link between MTK1 and actin has not been established in mammalian cells. Furthermore, even though HER2 and HER3 are involved in actin reorganization and cell migration, MTK1 has not been identified in this signaling process.

Cancer cells have increased glycolytic metabolism leading to acid loading and excess protons are excreted by up-regulating proton transporters (Stock et al., 2005). Heregulin stimulation of breast cancer cells leads to extracellular acidification of media that is dependent on HER2/HER3 activity (Chan et al., 1995). Additionally, extracellular acidification affects cell migration and invasion (Kim et al., 2007; Sennoune et al., 2004). For instance, human melanoma cells treated with acidic media excrete proteases required
for migration and are more invasive (Rofstad et al., 2006). With regard to HER2/HER3 signaling, although many signaling proteins have been linked to these receptors it is not clear how HRG regulates proton transporters.

Proteins that function in the MTK1 pathway have not been fully characterized nor has the regulation of MTK1 kinase activity. Previously we have shown regulation of mouse MAP3K4 (MEKK4) to be through activation of the IFNγ cytokine receptor (Halfter et al., 2005) and the GPCR for angiotensin II (Derbyshire et al., 2005). In this study we investigated whether MTK1 is also regulated by the activation of RTK’s in MCF-7 and T-47D epithelial breast cancer cells. We report the recruitment of MTK1 with only activated HER3 in response to HRG in both MCF-7 and T-47D cells. MTK1 is also required for HRG induced cell migration in MCF-7 breast cancer cells through the HER2/HER3 heterodimer. Additionally, HRG induces association of MTK1 with p85 of PI3K, likely via phosphoHER3. It has been reported that HRG stimulation leads to extracellular acidification (Chan et al., 1995) an event that is linked to cancer cell migration (Stock et al., 2005; Stock and Schwab, 2009). We demonstrate that knockdown of MTK1 inhibits HRG-induced extracellular acidification and cell migration. Furthermore, pre-treatment of MCF-7 cells with the HER2 kinase inhibitor lapatinib inhibits association of MTK1 and HER3. MTK1 also associates with actin through the actin interacting region (AIR) and disruption of the actin cytoskeleton using cytochalasin D inhibits MTK1 and HER3 association. Together, this report establishes
MTK1 as an integral signaling protein downstream of activated HER2 and HER3, required for acidification of the extracellular environment and cell migration.
2.2 MATERIALS AND METHODS

2.2.1 CELL CULTURE AND TREATMENTS

HEK-293, T-47D and MDA-MB-231 cells were cultured in Dulbecco’s modified Eagles medium with high glucose (DMEM) pH 7.4, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. MCF-7 cells were maintained in the same media as T-47D cells and supplemented additionally with 10 µg/ml insulin. Prior to experimental procedures, cells were cultured in DMEM supplemented only with 1% penicillin-streptomycin for 16 hrs. Cells were stimulated with 10 nM heregulin-β1 (HRG) EGF-Domain (Millipore Cat # 01-201) for 12 minutes unless otherwise indicated, EGF 3.3 nM for 12 minutes, 0.3 M sorbitol for 30 minutes or vehicle (30% glycerol in 1 x phosphate buffered saline pH 7.4) for 12 minutes. Pre-treatment with 250 nM lapatinib was performed during serum starvation for 16 hours unless otherwise indicated. Cells were treated with 1 µg/ml Cytochalasin D for 30 minutes prior to addition of HRG.
2.2.2 WESTERN BLOTTING AND ANTIBODIES

MCF-7 or T-47D cells were lysed in lysis buffer (70 mM β-glycerol phosphate, 1 mM EGTA, 1 mM dithiothreitol, 2 mM MgCl₂ and 0.5% Triton X-100) with protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride, 127.4 KIU/ml aprotinin (Calbiochem Cat # 616399), 10 µM leupeptin and with 0.5 mM sodium orthovanadate. Proteins were resolved by 5% – 12.5% gradient SDS-PAGE and transferred onto Protran 0.45 µm nitrocellulose blotting membrane (BioExpress Cat # F-3120-7). Membranes were blocked with 5% non-fat dry milk in 25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl and 0.15% Tween 20 (TBS-T). Immunostaining was performed in 5% non-fat dry milk in TBS-T and detected using chemiluminescence reagent (100 mM Tris pH 8.5, 250 mM luminol, 92 mM p-coumaric acid and 0.018% H₂O₂). Images were obtained using ChemiDoc™ XRS+ (BIO-RAD) and quantification was performed with Image Lab Software. After the initial immunoblots were performed, the nitrocellulose membranes were stripped at 56°C for 1 to hour using membrane stripping buffer (12.5 mM Tris pH 6.8, 2% SDS, 0.7% β-mercaptoethanol) to remove primary and secondary antibody. Membranes were re-imaged before additional immunoblots to ensure stripping was complete. Subsequent immunoblots were then performed the same way as described above. Antibodies were purchased from Cell Signaling (anti-mouse HRP-conjugated #7076S; anti-rabbit HRP-conjugated #7074S), Millipore (phosphotyrosine mouse monoclonal Clone 4G10 #05-321), Epitomics (EGFR #1902-1, HER2 #2064-1, HER3 #1186-1, HER4 #2218-1 and HER3 pY1289 #2526-1 rabbit monoclonal antibodies),
Santa Cruz Biotechnology (PI3-Kinase p85α mouse monoclonal #sc-1637), Thermo Scientific (actin mouse monoclonal #MA1-744), Sigma (Anti-FLAG mouse monoclonal #F1804) and MTK1 antibodies used were developed as we previously described (Derbyshire et al., 2005). All commercial antibodies were used according to manufacturer recommendations.

2.2.3 IMMUNOPRECIPITATION EXPERIMENTS

HEK-293, MCF-7, T-47D or MDA-MB-231 cells were stimulated with 10 nM HRG for 12 minutes, 0.3 M sorbitol for 30 minutes, 3.3 nM EGF for 12 minutes, 10 µg/ml insulin for 30 minutes or vehicle, unless otherwise indicated. The cells were then washed twice with ice cold 1X phosphate buffered saline pH 7.4. The cells were then lysed in lysis buffer and 2 mg of cell extract was immunoprecipitated for 1 hour at 4°C with 10 µg rabbit anti-MTK1 polyclonal antibody (Derbyshire et al., 2005); 4 µg mouse phosphotyrosine antibody (Millipore Clone 4G10 #05-321) or 20 µl anti-FLAG beads (Clontech #635686). Immune complexes recovered using protein A-Sepharose beads (Sigma Cat# P3391) were washed twice with ice cold lysis buffer and denatured with Laemmli sample buffer. Proteins were separated by 5–12.5% gradient SDS-PAGE and transferred to nitrocellulose membranes that were then subjected to immunoblot analysis as indicated.
2.2.4 CELL PROLIFERATION AND MEDIA PH MEASUREMENTS

MCF-7 and T-47D cells were seeded in complete growth media at a density of $0.4 \times 10^6$ cells per well in 12-well plates (VWR # 62406-165) for proliferation assays and $0.7 \times 10^6$ cells per well for pH measurements. Cells were allowed to adhere for 16 hours, followed by 4 hour serum starvation in DMEM, 1% penicillin-streptomycin without fetal bovine serum. The cells were then stimulated with 10 nM HRG, 3.3 nM EGF, 250 nM lapatinib (lapatinib pre-treatment was started at time of serum starvation) or vehicle for 24 hours in DMEM with 0.5% fetal bovine serum, 1.0% penicillin/streptomycin and 10 µg/ml human recombinant insulin in MCF-7 cells. Each condition was performed in triplicate and repeated a minimum of 3 times. After 24 hours, the cells were counted using a TC-10 Bio-Rad automated cell counter and the media pH was recorded using a pH meter probe. Data shown for each condition represents $n = 3$. A two sided student t-test with standard deviation were used to perform statistical analyses on proliferation rates and media pH measurements. Statistical significance was determined by a $p$-value $\leq 0.05$.

2.2.5 siRNA KNOCKDOWN EXPERIMENTS

MCF-7 cells were seeded (onto 10 cm cell culture plates, VWR # 353003) at a density of $5.0 \times 10^6$ cells and allowed to adhere for 16 hours. The cells were then transfected with 434 pmol of siRNA specific for MTK1 (Thermo Scientific ON-TARGETplus
SMARTpool, Human MAP3K4 (4216) Cat # L-003789-00-0005) or non-targeting (NS) (ON-TARGETplus Non-targeting Pool Cat # D-001810-10-05) as control using lipofectamine 2000 according to manufacture recommendations. The cells were transfected with siRNA a second time 24 hours later using lipofectamine 2000 to enhance knockdown efficiency. Twenty four hours after the second transfection the cells were detached using 0.25% trypsin (Invitrogen # 15050-065). The cells were counted and seeded onto 12 well plates (VWR # 62406-165) at a density of 0.7 x 10^6 cells per well for pH measurements and scratch assays or at density of 0.4 x 10^6 cells per well for proliferation assays. MTK1 knockdown efficiency was determined by immunoblot analysis using 150 µg whole cell lysate at 120 hours post-secondary transfection. Each condition was performed in triplicate and repeated a minimum of 3 times. Data shown for each condition represents n = 3. Statistical analysis was performed as described above.

2.2.6 SCRATCH ASSAY EXPERIMENTS

Scratch assays were performed in 12-well plates using MCF-7 cells at a density of 0.7 x 10^6 cells per well. The cells were allowed to adhere for 16 hours followed by 4 hour serum starvation in DMEM. The surface area of the cells was scratched using a 10 µl pipette tip (VWR # 89140-164) and the cells were then washed twice with 1 x phosphate buffered saline pH 7.4. The cells were then stimulated with 10 nM HRG or vehicle,
pretreated with or without 250 nM lapatinib (lapatinib pre-treatment was started at the time of serum starvation). Once scratches were performed and HRG stimulation was started, this was considered time zero and images were acquired using a 12.2 megapixel digital camera (GE model # W1200) visualized through a Leica Microsystems inverted microscope (model # DM IL) at 10 x magnification. Additional images were taken at 24 and 48 hours and measurements were taken from 24 hours and 48 hours and compared to time zero for each condition. Each condition was performed in triplicate and repeated a minimum of 3 times. Data shown for each condition represents n = 3. Statistical analysis was performed as described above.
2.3 RESULTS

2.3.1 ASSOCIATION OF A 180 KDA TYROSINE PHOSPHORYLATED PROTEIN WITH MTK1

To investigate RTK regulation of MTK1 we selected MCF-7 and T-47D breast cancer epithelial cells, which express moderate levels of the ErbB RTK receptors 1-4 (Beerli and Hynes, 1996; Carraway et al., 1994). Heregulin β1 (HRG) was used to stimulate these cells because HER3 and HER4 bind HRG allowing homo and hetero dimerization between HER1-4 (Aguilar et al., 1999; Carraway and Cantley, 1994; Neve et al., 2002). MCF-7 or T-47D cells were stimulated with HRG or sorbitol. Sorbitol was used to induce osmotic stress and activate MTK1, since MTK1 is known to function in the p38 MAP kinase pathway (Abell et al., 2007; Aissouni et al., 2005; Takekawa and Saito, 1998). After stimulation, MTK1 was immunoprecipitated with MTK1 antibody that recognizes the amino-terminal proline-rich region of MTK1 (Figure 3A). Immunoprecipitated MTK1 and associated proteins were resolved by SDS-PAGE, followed by transfer to nitrocellulose membrane and immunobloting for tyrosine phosphorylation (pTyr). A tyrosine phosphorylated protein co-immunoprecipitated with MTK1 in response to HRG stimulation (Figure 3B, top panel, lane a), which was not present in cells stimulated with vehicle or sorbitol (Figure 3B, top panel, lanes b and c). The tyrosine phosphorylated protein had a molecular weight of 180 kDa, which is approximately the same molecular weight as MTK1. This result suggested that MTK1
may be phosphorylated on tyrosine in response to HRG stimulation, or that a HER family member co-precipitated with MTK1.

2.3.2 HER3 ASSOCIATES WITH MTK1 IN RESPONSE TO HRG STIMULATION

We set out to investigate the identity of the 180 kDa protein. HER1-4 were candidate proteins for tyrosine phosphorylation and association with MTK1, since HER3 and HER4 bind HRG and can trigger homo and hetero-dimerization of these four receptors (Britsch, 2007; Yarden and Sliwkowski, 2001). T-47D cells were stimulated with HRG and cell extracts were prepared in which MTK1 was immunoprecipitated in four different samples. The precipitated proteins were resolved by SDS-PAGE and immunoblotted for HER1-4 to identify which of the HER proteins associates with MTK1. HER3 was identified as associating with MTK1 in response to HRG stimulation (Figure 3D, top panel, lane a). The fact that immunoblotted HER3 from the immunoprecipitation migrated slower than the proteins from the cell lysates suggested that phosphorylated HER3 preferentially interacted with MTK1. EGFR and HER2 did not associate with MTK1 in response to HRG or vehicle stimulation (Figure 3C, top panel, lanes a & b, and lanes e & f). Moreover, HER4 did not associated with MTK1 in response to HRG (Figure 3D, top panel, lanes e & f), even though HER4 binds HRG (Plowman et al.,
1993). Immunoprecipitation of MTK1 was validated for each experimental condition by immunobloting for MTK1 (Figure 3C and Figure 3D, bottom panels).
FIGURE 3. HER3 ASSOCIATES WITH MTK1 IN RESPONSE TO HRG STIMULATION.

A schematic representation of the proline-rich region (PPP), actin-interacting region (AIR), alanine-rich region (AAA), and catalytic domain of MTK1 is shown. The MTK1 antibody recognizes an amino terminal region (amino acids 18–139) of MTK1 (Panel A). T-47D cells were stimulated with 10 nM HRG for 12 minutes or 0.3 M sorbitol for 30 minutes as indicated (Panel B). MTK1 was immunoprecipitated and proteins were resolved by SDS-PAGE and immunoblotted using monoclonal antibody 4G10 (pTyr). T-47D cells were stimulated with HRG and MTK1 was immunoprecipitated as described above (Panels C & D). Immunoblots were performed using antibodies directed against EGFR and HER2 (Panel C) or HER3 and HER4 (Panel D). Membranes were stripped and immunoblotted with MTK1 antibody (B, C and D, bottom panels).
2.3.3 MTK1 IS NOT TYROSINE PHOSPHORYLATED IN RESPONSE TO HRG

Our results suggested that HER3 was the tyrosine phosphorylated protein that interacts with MTK1. However, the results did not exclude the possibility that MTK1 was phosphorylated on tyrosine especially since we were immunoprecipitating MTK1 and it has a similar molecular mass as the tyrosine phosphorylated protein. If MTK1 was tyrosine phosphorylated, we should be able to immunoprecipitate MTK1 with an antibody specific for phospho-tyrosine. We stimulated T-47D cells with HRG and performed an immunoprecipitation with monoclonal antibody 4G10 that recognizes tyrosine phosphorylation. A protein of 180 kDa was immunoprecipitated with the 4G10 monoclonal antibody and the protein was phosphorylated on tyrosine (Figure 4A, top panel, lane a). Similarly, a 180 kDa tyrosine phosphorylated protein was present in the MTK1 immunoprecipitation (Figure 4A, top panel, lane c) When normal rabbit IgG was used as a control during the immunoprecipitation, no tyrosine phosphorylated proteins were detected in response to HRG (Figure 4A, top panel, lane e). The membrane was stripped and immunoblotted for MTK1. The results revealed that MTK1 was not present in the phosphotyrosine immunoprecipitation (Figure 4A, bottom panel, lanes a & b), which strongly suggests that MTK1 is not tyrosine phosphorylated in response to HRG. As expected, MTK1 was present in the MTK1 immunoprecipitation (Figure 4A, bottom panel, lanes c & d). Additionally, normal rabbit IgG did not immunoprecipitate MTK1 (Figure 4A, bottom panel, lanes e & f).
2.3.4 PHOSPHOHER3 ASSOCIATES WITH MTK1 IN RESPONSE TO HRG

HER3 has a molecular mass of 180 kDa and among many phosphotyrosines there are several YXXM motifs that are phosphorylated in response to HRG (Hellyer et al., 1998). There are commercial phospho-antibodies that recognize several of these phosphorylation sites, one of which is pY1289. In order to determine if MCF-7 cells behave similarly to T-47D cells, we used MCF-7 cells in our next experiment. We stimulated MCF-7 cells with HRG and performed an immunoprecipitation with monoclonal antibody 4G10. HER3 was immunoprecipitated with the 4G10 monoclonal antibody and was phosphorylated on Y1289 (Figure 4B, top panel, lane g). Similarly when a HER3 antibody was used for immunoprecipitation, phosphoHER3 was detected with the pY1289 phospho antibody (Figure 4B, top panel, lane i). Additionally, HER3 co-precipitated with MTK1 when the immunoprecipitation was performed with MTK1 antibody and HER3 was tyrosine phosphorylated on Y1289 (Figure 4B, top panel, lane k). The membrane was stripped, immunoblotted for MTK1, and MTK1 was not detected in the phosphotyrosine immunoprecipitation (Figure 4B, bottom panel, lanes g & h). These results demonstrate that HRG regulates the association of MTK1 with activated HER3 and that MTK1 is not phosphorylated on tyrosine in both T-47D and MCF-7 breast cancer cells.
Additionally, the University of Arizona Southwest Environmental Health Sciences Center (SWEHSC) proteomics core facility was used to further characterize the association between MTK1 and HER3 in response to HRG stimulation. A series of six MTK1 immunoprecipitations was performed in which 2 mg of cell extract was used for each immunoprecipitation. After washing the immunoprecipitations with lysis buffer, the samples were pooled and resolved in one lane by SDS-PAGE followed by silver staining. Silver stained proteins were aligned with a replicate experiment that was immunoblotted with phosphotyrosine antibody (Figure 4C, lane a). The area of the gel aligning with the 180 kDa tyrosine phosphorylated protein was excised (Figure 4C, lanes c & d). The proteins were digested with trypsin, separated and analyzed via Orbitrap LC-MS. HER3 was identified as associating with MTK1 in response to HRG with 26% of the HER3 protein sequenced and several of the peptides identified with a 99.9% degree of confidence (Figure 4D, right panel). Several of these peptides were sequenced multiple times increasing confidence for positive identification of HER3. Proteomic analysis did not identify HER3 as associating with MTK1 in response to vehicle stimulation. This result is consistent with our immunoblot analysis which suggests that the association between HER3 and MTK1 is not constitutive, but requires HRG stimulation.
FIGURE 4. HER3 ASSOCIATES WITH MTK1 IN BOTH T-47D AND MCF-7 CELLS.

Cells were stimulated as described in Figure 1. Cell lysates were incubated with antibody directed against phosphotyrosine (pTyr), MTK1, or normal rabbit IgG. Proteins were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (A, top...
panel) or antibody specific for phosphotyrosine 1289 of HER3 (B, top panel). The membranes were re-probed for MTK1 (bottom panels). MCF-7 cell lysates were prepared as described above and a fraction of the immunoprecipitate (15%) was immunoblotted using anti-phosphotyrosine antibody (Panel C, lanes a & b), while the remaining immunoprecipitation was silver stained (lanes c & d). HER3 peptides were identified in the HRG stimulated sample (arrows) by LC-MS/MS with 99.9% confidence (D).
Although HER3 peptides were not identified from the MTK1 immunoprecipitation of vehicle stimulated MCF-7 cells, MTK1 peptides were identified because MTK1 and HER3 migrate at similar positions by SDS-PAGE (Fig 5). MTK1 peptides were identified from both the HRG and vehicle stimulated conditions. A total of 56% of the MTK1 protein was identified as tryptic peptides with a 99.9% degree of confidence for positive identification of MTK1. The identification of MTK1 peptides and not HER3 peptides from the vehicle stimulated sample indicates that the lack of HER3 peptides in this sample was not a technical artifact. In addition, there were no tyrosine phosphorylated peptides identified for MTK1 in either HRG or vehicle stimulated conditions which is consistent with the immunobloting data.
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FIGURE 5. HER3 AND MTK1 PEPTIDES IDENTIFIED BY MASS SPECTROMETRY.

Tryptic peptides were obtained from the MTK1 immunoprecipitation experiment shown in figure 2C after silver staining the gel. The confidence level of positive protein identification was determined as high, medium or low by using the Proteome Discoverer software (Thermo Fisher Scientific). A confidence level of 99.9% or greater was deemed as high confidence; a confidence level above 95% was deemed as medium confidence; and a confidence level of less than 95% was deemed as low confidence. HER3 peptides from HRG stimulation followed by MTK1 immunoprecipitation are in the left column. MTK1 peptides from HRG or vehicle (no HRG) stimulation followed by MTK1 immunoprecipitation are in the middle and right columns, respectively. There were no HER3 peptides identified in vehicle stimulated MTK1 immunoprecipitation.
A time course experiment was performed in which MCF-7 cells were stimulated with HRG for as long as 150 minutes. In other experiments, the tyrosine phosphorylation of the 180 kDa protein associating with MTK1 occurred within 30 seconds (data not shown). In longer time course experiments, the tyrosine phosphorylated protein that co-immunoprecipitated with MTK1 was observed from 5 for up to 150 minutes after HRG stimulation (Figure 6A, upper panel, lanes b-j). In HRG washout experiments where HRG was washed out after each time point, the tyrosine phosphorylation was more transient (data not shown). These experiments demonstrate that constant HRG exposure results in continued HER3 activation and association with MTK1. This result may be significant in breast cancer cases in which HRG is over-expressed.

2.3.5 HER3 AND MTK1 ASSOCIATION IS SPECIFIC TO HRG STIMULATION

In order to determine whether other receptor tyrosine kinases interacted with MTK1, T-47D cells were stimulated with EGF or insulin and then MTK1 was immunoprecipitated as described above. As a positive control, cells were stimulated with HRG. Immunoblot analysis using 4G10 mouse monoclonal antibody revealed a protein of approximately 150 kDa that was tyrosine phosphorylated in response to insulin (Figure 6B, top panel, lane d). No tyrosine phosphorylated proteins immunoprecipitated with MTK1 after stimulation with EGF, sorbitol or vehicle (Figure 6B, top panel, lanes c, e & f). Since
EGF can activate the EGFR and HER2, the lack of tyrosine phosphorylated proteins indicates that neither EGFR nor HER2 interact with MTK1, while HRG selectively activates HER3 to interact with MTK1 (lane b). The ~150 kDa tyrosine phosphorylated protein that immunoprecipitated with MTK1 in response to insulin was later identified as insulin receptor substrate-1 (IRS-1; data not shown). The membrane was stripped and immunoblotted for MTK1 (Figure 6B, middle panel). To establish stimulation efficacy, lysates from vehicle, HRG, EGF, insulin and sorbitol were immunoblotted with G410 mouse monoclonal antibody (Figure 6B, top panel lanes a & g-j).

2.3.6 P85 OF PI3K ASSOCIATES WITH MTK1

HER3 has several YXXM motifs which, when phosphorylated, function as recruitment sites for the SH2 domain of the p85 regulatory subunit of PI3K (Hellyer et al., 1998). Therefore, it was possible that p85 would co-precipitate with MTK1 in response to HRG. p85 of PI3K was identified as immunoprecipitating with MTK1 in response to HRG stimulation (Figure 6B, bottom panel, lane b). Additionally, p85 was also shown to immunoprecipitate with MTK1 in response to insulin stimulation, likely through an interaction with IRS (Figure 6B, bottom panel, lane d). These results demonstrate that the HER3/MTK1 complex also includes PI3K suggesting that the HER3/MTK1 complex utilizes 3’ phosphorylated inositol phosphates as part of the signaling mechanism.
MCF-7 cells were stimulated with 10 nM HRG for the indicated times. MTK1 was immunoprecipitated and proteins were resolved by SDS-PAGE, then immunoblotted with anti-phosphotyrosine antibody (A, top panel). The membrane was re-robed with MTK1 antibody (bottom panel). T-47D cells were stimulated with 10 nM HRG for 12 minutes, 3.3 nM EGF for 12 minutes, 10 µg/ml insulin for 15 minutes or 0.3 M sorbitol for 30 minutes followed by immunoprecipitation of MTK1. The proteins were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.
2.3.7 HER2 IS REQUIRED FOR CO-IMMUNOPRECIPITATION OF PHOSPHORYLATED HER3 WITH MTK1

HER3 does not have intrinsic kinase activity and is phosphorylated by heterodimerization with either HER1, 2 or 4 (Stern, 2008). HER2, HER3 and HER4 are tyrosine phosphorylated in MCF-7 cells stimulated with HRG (data not shown). Thus, HER2 and HER4 are possible candidates for phosphorylating HER3, since HER2/HER3 and HER3/HER4 heterodimers are formed in response to HRG. Since HER2 and HER3 are preferred heterodimers (Way and Lin, 2005), HER2 was hypothesized as the most likely candidate for phosphorylation of HER3. MCF-7 cells were pretreated with lapatinib to inhibit the kinase activity of HER2 and then stimulated with HRG followed by immunoprecipitation of MTK1. Lapatinib has a $K_i$ of 13 nM for inhibition of HER2 (Wood et al., 2004). Lapatinib decreased the interaction between HER3 and MTK1 in a dose dependent manner as evidenced by decreased tyrosine phosphorylation of HER3 (Figure 7A). These results indicate that HER2 catalytic activity is required for HER3 phosphorylation and subsequent interaction with MTK1 in response to HRG. The membrane was stripped and immunoblotted for MTK1 (Figure 7A, bottom panel). Lysates from the same experiment show tyrosine phosphorylation of HER3 is decreased in response to lapatinib (Figure 7B, top panel, lanes c-e).
MDA-MB-231 breast cancer cells were used to further characterize HER2 involvement in the formation of the HER3-MTK1 protein complex in response to HRG. MDA-MB-231 cells are a triple negative cell line and do not express estrogen receptor, progesterone receptor or HER2, but do express HER3 (Teixido et al., 2012). If HER2 is required for HER3 phosphorylation and association with MTK1, then we should not see the association of HER3 with MTK1 in MDA-MB-231 cells. T-47D, MDA-MB-231 and MCF-7 cells were stimulated with HRG followed by immunoprecipitation of MTK1. Immunoblot analysis using 4G10 mouse monoclonal antibody revealed that a 180 kDa tyrosine phosphorylated protein immunoprecipitated with MTK1 in T-47D and MCF-7 cells dependent on HRG (Figure 7C, top panel, lanes a & e). No tyrosine phosphorylated proteins immunoprecipitated with MTK1 from the MDA-MB-231 cells (Figure 7C, top panel, lanes c & d), which strongly suggests that HER2 is required for formation of the HER3 MTK1 complex. The membranes were stripped and immunoblotted for MTK1 to demonstrate that MTK1 is expressed in in MDA-MB-231 cells (Figure 7C, bottom panel).
FIGURE 7. HER2 KINASE ACTIVITY IS REQUIRED FOR MTK1/HER3 ASSOCIATION.

MCF-7 cells were pre-treated with 0, 50, 100 or 200 nM lapatinib for 1 hour followed by 10 nM HRG stimulation for 12 minutes. Phosphotyrosine immunoblot analysis of MTK1 immunoprecipitations show 50 nM lapatinib attenuates MTK1/HER3 association (Panel A, lane c). Cell extracts (150 µg) that were used for the immunoprecipitation were resolved by SDS-PAGE and immunoblotted for phosphotyrosine (B, top panel). HER2 negative, but HER3 positive MDA-MB-231 cells were stimulated with 10 nM HRG followed by MTK1 immunoprecipitations and compared to T-47D and MCF-7 cells. Phosphotyrosine immunoblot analysis shows HER2 is required for MTK1/HER3 association in response to HRG (Panel C, lane c). Membranes were stripped and immunoblotted for MTK1 (A, B and C, bottom panels).
2.3.8 ACTIN INTERACTING REGION (AIR) OF MEKK4 IS REQUIRED FOR ACTIN ASSOCIATION WITH MEKK4

Human MTK1 and the mouse homolog, MEKK4, have similar homology to the yeast protein, Ssk2p (Bettinger and Amberg, 2007). Ssk2p has an actin interacting region (AIR) that is required for actin cytoskeleton recovery after osmotic stress (Yuzyuk et al., 2002b). Alignment of the AIR of Ssk2p with MEKK4 shows 17/40 amino acids are identical between these proteins and 25/40 are highly conserved, suggesting that MEKK4/MTK1 might interact with actin (Figure 8A). The AIR of MEKK4 consisting of amino acids 256-295 was deleted from the MEKK4 cDNA. Flag-tagged wild-type MEKK4 and flag-tagged AIR MEKK4 were transfected into HEK 293 cells. After 48 hours, the HEK cells were serum starved for 4 hours followed by stimulation with 20% fetal bovine serum or sorbitol as possible inducers of the MTK1-actin interaction. MEKK4 was immunoprecipitated with a flag antibody-coupled to beads that specifically recognizes the flag epitope. Actin did not associate with MEKK4 when the AIR was deleted (Figure 8B, second panel, lanes g-i), while actin constitutively associated with wild-type MEKK4 independent of stimulation (Figure 8B, second panel, lanes d-f). These results demonstrate that MEKK4/MTK1 behave like Ssk2p and interact with actin within a unique 40 amino acid region.
2.3.9 DISRUPTION OF THE ACTIN CYTOSKELETON SIGNIFICANTLY DECREASES THE ASSOCIATION BETWEEN HER3 AND MTK1

HRG enhances the conversion of g-actin to f-actin leading to increased cell migration (Adam et al., 1998). Since HER3 is regulated by HRG, the association between MTK1 and HER3 that we observed may be facilitated by actin in response to HRG. To test this hypothesis, MCF-7 cells were pre-treated with cytochalasin D to disrupt actin polymerization and the formation of f-actin. The cells were then stimulated with HRG or sorbitol followed by immunoprecipitation of MTK1 and pY1289 HER3 immunoblots. In the presence of cytochalasin D, the interaction between HER3 and MTK1 was diminished by 60% (Figure 8C, top panel, compare lanes a & d). Interestingly, phosphorylation of HER3 remained the same in the HRG stimulated lysates (Figure 8C, top panel, lanes g & j). These data suggest that f-actin is required for the association between activated HER3 and MTK1 in response to HRG, but f-actin is not required for HRG-dependent activation of HER3.
FIGURE 8. ACTIN ASSOCIATES WITH MTK1 AND INHIBITION OF ACTIN POLYMERIZATION WITH CYTOCHALASIN D INHIBITS MTK1/HER3 ASSOCIATION.

Comparison of mouse MTK1 and the actin interacting region (AIR) of the yeast homolog Ssk2p shows 17/40 amino acids are identical and 25/40 are conserved (Panel A). Amino acids 256-295 were deleted from the mouse MTK1 cDNA (AIR mutant). HEK-293 cells were transfected with empty vector, flag-tagged wild-type mouse MTK1 or the flag-tagged AIR mutant mouse MTK1 cDNA. After 48 hours, the cells were stimulated with 20% fetal bovine serum or 0.3M sorbitol. Flag antibody-beads were used for immunoprecipitation for flag-tagged proteins. Proteins were resolved by SDS-PAGE and immunoblotted with antibody that recognizes the FLAG epitope (B, top panel) or actin (lower panel). Cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. MCF-7 cells were pre-treated with 1 µg/ml cytochalasin D (Cyto D) for 30 minutes followed by 10 nM HRG for 12 minutes or 0.3M sorbitol for 30 minutes. MTK1 was then immunoprecipitated from each condition (C, lanes a-f). Antibody directed against phosphotyrosine 1289 of HER3 was used to immunoblot (top panel). The membrane was re-probed for MTK1 (bottom panel). Cell lysates were resolved by SDS-PAGE and immunoblotted as indicated. Cytochalasin D had no effect on the phosphorylation of HER3 (lanes g & j), but diminished interaction between MTK1 and HER3 (lanes a & d).
2.3.10 MTK1 IS REQUIRED FOR HRG-INDUCED EXTRACELLULAR ACIDIFICATION

Since HRG is known to induce proliferation, invasion, migration and metastasis (Asrani et al., 2013; Atlas et al., 2003; Fiddes et al., 1995; Hernandez et al., 2009; Xue et al., 2006; Yang et al., 2008), it was necessary to identify the specific functional role of HRG in MCF-7 breast cancer cells. Assays were performed to determine if HRG stimulation leads to proliferation of MCF-7 cells. MCF-7 cells were stimulated with HRG or EGF and after 24 hours the cells were counted using a Bio-Rad TC-10 automated cell counter in the presence of trypan blue. EGF, unlike HRG, induced cell proliferation (Figure 9A, left panel). There was no difference in cell viability between each condition (data not shown). However, there was a noticeable difference in the color of the DMEM media after 24 hours of HRG stimulation, which was not observed with EGF stimulation. Media from the HRG stimulated cells appeared orange, which is an indicator of increased acidity or decreased pH. Measurements of the media pH revealed that HRG stimulation led to extracellular acidification of the cell culture media when compared to vehicle (Figure 9A, right panel). EGF stimulation did not induce extracellular acidification (Figure 9A, right panel). Similarly, T-47D cells stimulated with HRG also had extracellular acidification independent of cell proliferation (data not shown).
2.3.11 MTK1 KNOCKDOWN INHIBITS HRG INDUCED EXTRACELLULAR ACIDIFICATION

HRG induced extracellular acidification and association of tyrosine phosphorylated HER3 with MTK1. These results suggest that MTK1 is required for HRG-induced proton excretion from the cell. To test this hypothesis MCF-7 cells were double transfected with siRNA that specifically targets MTK1 and non-specific (NS) siRNA as a control (double transfections were performed to increase transfection efficiency). The cells were stimulated with HRG and after 24 hours, media was collected and pH measurements were recorded with a pH meter probe. Knockdown of MTK1 inhibited HRG induced extracellular acidification when compared to NS siRNA and non-transfected cells (Figure 9B, lanes b, d & f). In addition, pre-treatment of MCF-7 cells with lapatinib for 30 minutes prior to stimulation with HRG resulted in inhibition of HRG-induced extracellular acidification (Figure 9B, lanes g & h) indicating a requirement for HER2 in the acidification response. Together, these results show that HER2 and MTK1 are required for HRG-induced extracellular acidification.

Knockdown of MTK1 could affect the proliferation rate resulting in fewer cells excreting protons. Therefore, proliferation assays were performed to rule out whether MTK1 knockdown had an effect on proliferation rates. MCF-7 cells were transfected with siRNA, as described previously and stimulated with HRG. After 48 hours the cells were
counted using a Bio-Rad TC-10 automated cell counter. Knockdown of MTK1 did not have an effect on the proliferation rate when compared to NS siRNA knockdown (Figure 9C, lanes a-d). Knockdown of MTK1 did not have an effect on viability either (data not shown). Since all of the experimental conditions shown in figure 9C started with the same amount of cells, it appears that transfection of MCF-7 cells did have an effect on proliferation rate when compared to non-transfected cells (Figure 9C, lanes e & f). However, the decreased proliferation rate was independent of MTK1 siRNA but dependent on transfection, likely reflecting the difficulty associated with transfecting MCF-7 cells.
Figure 9

A

24 hr Proliferation Assay

Cells (x10^6)

Vehicle HRG EGF

pH Measurements

Media pH

Vehicle HRG EGF

B

Media pH

* * *

a b c d e f g h

MTK1 siRNA NS siRNA Non-transfected Lapatinib

C

48 hour Proliferation Assay

Cells (x10^6)

Vehicle HRG

a b c d e f

MTK1 siRNA NS siRNA Non-transfected
FIGURE 9. MTK1 IS REQUIRED FOR HRG INDUCED EXTRACELLULAR ACIDIFICATION.

MCF-7 cells were stimulated with 10 nM HRG or 3.3 nM EGF, after 24 hours the cells were counted (Panel A, left graph) and the pH of the media was measured (right graph). MTK1 siRNA knockdown was used to determine whether MTK1 is required for HRG induced extracellular acidification. MCF-7 cells were transfected as described in the methods section. The cells were then stimulated with 10 nM HRG for 24 hours followed by pH measurements of the media (Panel B). Non-transfected or cells pre-treated with 250 nM lapatinib were also stimulated with HRG followed by pH measurements (Panel B). MTK1 knockdown did not have an effect on cell proliferation compared to NS knockdown (Panel C). Statistical analysis was performed using a two-sided student t-test with standard deviations and a minimum of three replicates, p-value of $\leq 0.05$ was considered statistically significant and each experiment was repeated a minimum of three independent times.
2.3.12 MTK1 IS REQUIRED FOR HRG INDUCED CELL MIGRATION

Cancer cells have increased glycolytic metabolism leading to acid loading and excess protons are excreted by up-regulating proton transporters (Stock et al., 2005). Migration is a possible functional response to HRG stimulation because acid excretion creates microextracellular environments that are favorable for cell migration (Stock and Schwab, 2009). HRG is also linked to intracellular actin cytoskeletal reorganization that leads to increased migration (Adam et al., 1998). Furthermore, MTK1 may have a role in HRG induced cell migration since MTK1 knockdown blocks extracellular acidification. To determine whether MTK1 is required for cell migration, HRG-induced scratch assays were performed with and without MTK1 knockdown. MCF-7 cells were transfected with MTK1 and NS siRNA as described above. MCF-7 cell transfections were performed using 10 cm culture dishes and then the cells were transferred to a 12-well plate after a 24 hour post-transfection recovery period. The surface of the well was scratched followed by HRG stimulation and digital image acquisition at 0, 24 and 48 hours post scratch. After 24 hours of HRG stimulation, non-transfected cells closed the scratched area by 80% (Figure 10A, column b). Cells pretreated with lapatinib only closed the scratched area by 27%. Similarly, cells transfected with MTK1 siRNA closed the scratched area by approximately 17% while the cells transfected with NS siRNA had closed the scratched area by approximately 60% (Figure 10A, column b). Additionally, MTK1 knockdown or lapatinib pretreatment continued to severely impair cell migration into the scratched area after 48 hours (Figure 10A, column c), while the cells transfected with NS siRNA had
almost completely migrated into the scratched area, much like the non-transfected cells (Figure 10A, top row). Statistical analysis revealed that MTK1 knockdown had a mean of 17.3% ± 4.6 cell migration (Figure 10B, lane b) and cells pretreated with lapatinib had a mean of 27.8% ± 4.4 cell migration (Figure 10B, lane d) compared NS knockdown mean of 55.3% ± 6.4 (Figure 10B, lane c). Similar results were observed at 48 hours with MTK1 knockdown cells migrating by a mean of 40.1% ± 6.8 (Figure 10C, lane b) and lapatinib treated cells migrating by a mean of 31.3% ± 2 (Figure 10C, lane d) compared to NS knockdown migrating by a mean of 89% ± 7.8 (Figure 10C, lane c). Very little cell migration was observed in cells not stimulated with HRG. Therefore, scratch assays of cells transfected with MTK1 siRNA or NS siRNA, and non-transfected cells all looked similar (only MTK1 siRNA is shown, Figure 10A bottom panel). MTK1 protein expression levels were measured 48 hours after scratch by immunoblot analysis. MTK1 protein expression was knocked down by 58% compared to NS siRNA (Figure 10D, top panel, lanes b & c). Actin was used as a loading control (Figure 10D, bottom panel). Together these results demonstrate that HRG-induced cell migration requires MTK1.
FIGURE 10. MTK1 IS REQUIRED FOR HRG INDUCED CELL MIGRATION.

MCF-7 cells were transfected as described previously. The cells were serum starved for four hours, then scratched followed by HRG stimulation. Digital images were acquired at
0, 24 and 48 hours post scratch (Panel A). Statistical analysis revealed MTK1 knockdown cells migrated by a mean of 17.3% ± 4.6 (Panel B, lane b) and cells pretreated with lapatinib had a mean of 27.8% ± 4.4 cell migration (Figure 7B, lane d) compared to a NS knockdown mean of 55.3% ± 6.4 (Panel B, lane c). Similar results were observed at 48 hours with MTK1 knockdown migrating by a mean of 40.1% ± 6.8 (Panel C, lane b) and lapatinib treated cells migrating by a mean of 31.3% ± 2 (Figure 7C, lane d) compared to NS knockdown by a mean of 89% ± 7.8 (Panel C, lane c). Statistical analysis was performed as described in figure 6. To measure knockdown efficiency of MTK1, cell extracts (150 µg) were resolved by SDS-PAGE, followed by MTK1 immunoblot analysis and densitometry measurements using BioRad Image Lab software to detect extent of MTK1 knockdown (Panel D, lane b), actin was used as a loading control (bottom panel).
2.4 DISCUSSION

Prior studies have used ectopic expression of MEKK4/MTK1 in yeast (Takekawa et al., 1997), over-expression of wild-type or dominant-negative mutants in mammalian cells (Gerwins et al., 1997; Kanungo et al., 2000; Mita et al., 2002; Stevens et al., 2006; Takekawa and Saito, 1998), or manipulation of MEKK4 genomic DNA (Abell et al., 2005; Sarkisian et al., 2006; Sun et al., 2011) as a means to study MEKK4/MTK1 function. In this study we have focused on the scaffolding properties of endogenous MTK1. We identify a heregulin-dependent recruitment of tyrosine phosphorylated HER3 to MTK1. HER2 kinase activity is required for the formation of the HER3/MTK1 heterodimer, which is stable and involves only tyrosine phosphorylated HER3. These results have clinical implications in patients prescribed drugs like trastuzumab and lapatinib to inhibit HER2 activity, since these drugs will more than likely indirectly inhibit the activity of the phosphoHER3/MTK1 heterodimer. We demonstrate that extracellular acidification and cell migration require MTK1. Furthermore, lapatinib inhibited extracellular acidification and cell migration. Thus, both normal and malignant biological processes dependent on extracellular acidification and cell migration would likely be inhibited in patients treated with trastuzumab or lapatinib.

Although it is not clear how these proteins interact, it is clear that tyrosine phosphorylated HER3 preferentially associates with MTK1 as demonstrated when the
hyper-phosphorylated, slower migrating form of HER3 co-immunoprecipitates with MTK1 (Figure 3D, top panel, lane a). Consistent with the immunobloting results, multiple peptides were identified by LC-MS/MS from heregulin-stimulated HER3, but no peptides were detected from the control sample, again demonstrating that only activated HER3 interacts with MTK1. These results strongly suggest that a specific or multiple phosphotyrosines mediate the interaction between phosphoHER3 and MTK1. MTK1 has no known SH2 domains so a direct interaction between MTK1 and phosphoHER3 seems unlikely. However, Shc, PI3K, and Grb7 have SH2 domains and are examples of proteins that interact with specific phosphotyrosines on HER3 (Jones et al., 2006) and these proteins could mediate the interaction between MTK1 and phosphoHER3.

The HER2/HER3 heterodimer has been reported as an oncogenic unit that drives breast tumor cell proliferation. Although HER2 is the only catalytically active kinase within the heterodimer, HER3 is required for breast tumor formation (Garrett et al., 2011; Holbro et al., 2003a; Vaught et al., 2012). Thus even though HER3 lacks tyrosine kinase activity and depends on HER2 for phosphorylation, the proteins that “decorate” phosphoHER3 are also mediators of HER2/HER3 tumorigenicity. We show for the first time that HRG stimulation recruits MTK1 to HER3, which is sustained for up to 150 minutes indicating that sustained exposure to HRG results in continuous signaling through MTK1 and does not result in receptor down-regulation, consistent with previous findings demonstrating lack of receptor down-regulation with HER2 and HER3 (Baulida et al., 1996). The ability of HER2/HER3 to continue signaling through MTK1 in the presence of HRG may
have clinical implications for patients with HRG-driven tumors as opposed to tumors with HER2 gene amplification. In tumors with over-expression of HRG, an autocrine or paracrine loop may play a role in cellular transformation and tumor progression that does not require HER2 over-expression. Identification of the HER3 regulated MTK1 pathway may provide a new molecular target for clinical intervention under conditions of normal levels of HER2/HER3 expression.

We also investigated whether MTK1 interacts with other RTKs. Although HRG also binds to HER4 (Hynes and Lane, 2005), phosphoHER4 does not appear to interact with MTK1 (Figure 1D). EGF is also an activator of HER2 (Khazaie et al., 1993), but stimulation of MCF-7 cells did not result in the recruitment of EGFR or HER2, or any other tyrosine phosphorylated proteins that would migrate at the position of 150 kDa or greater (Figure 6B, lane c). Insulin stimulation of MCF-7 cells led to the recruitment of a 150 kDa tyrosine phosphorylated protein (Figure 6B, lane d). We identified this protein as the insulin receptor substrate-1. When the insulin receptor is activated, IRS-1 associates with the insulin receptor and is tyrosine phosphorylated. IRS-1 helps attenuate insulin signaling in healthy individuals (Schmitz-Peiffer and Whitehead, 2003). However, IRS-1 is also associated with breast cancer and is characterized as a transforming oncogene (Chan and Lee, 2008). The interaction of MTK1 with HER3 and IRS-1 strongly links MTK1 to RTK signaling. Thus, the interaction between MTK1 and IRS-1 may be a potential new avenue to explore in breast cancer therapy. Finally, stimulating MCF-7 cells with sorbitol did not result in the recruitment of phosphoHER3
with MTK1 indicating that the interaction between MTK1 and phosphoHER3 is not related to osmotic stress (Figure 6B, lane e). In summary, the recruitment of MTK1 with phosphoHER3 appears to be unique to HRG binding to the HER2/HER3 heterodimer identifying an entirely new signaling pathway downstream of HER3.

The carboxyl-terminus of HER3 contains six YXXM motifs that when phosphorylated allow association of p85 of PI3K (Hellyer et al., 1998; Soltoff et al., 1994). Mutation of the YXXM motifs from tyrosine to phenylalanine inhibits HRG-induced migration suggesting that recruitment of PI3K with HER3 is important in mediating cell migration (Smirnova et al., 2012). Furthermore, inhibition of PI3K with PIK-75 inhibits cell motility and invasion (Smirnova et al., 2012). We report that knockdown of MTK1 also blocks HRG-induced migration (Figure 10). It is tempting to speculate that a single mole of phosphoHER3 and MTK1 functions as a signaling complex to recruit as many as six moles of PI3K, leading to amplification of the cell migration signal. Thus even though MTK1 knockdown was not highly efficient, loss of 60% of MTK1 expression was sufficient to prevent HRG-dependent cell migration. Perhaps indicating that a threshold of PI3K activity cannot be mobilized to lamellipodia or other cellular regions actively involved in cell migration. Similarly in the presence of insulin, PI3K is recruited to MTK1 (Figure 6B, lane d), likely due to the presence of IRS-1 which is known to associate with PI3K (Kooijman et al., 1995). The relationship between MTK1 and insulin in terms of cellular response remains to be explored, especially in regards to glucose homeostasis.
We also demonstrate that HER3/MTK1 association requires HER2 kinase activity, but HER2 does not remain in the complex. Lapatinib inhibits EGFR, HER2 and HER4 by binding to the ATP-binding pocket of these kinases, thereby inhibiting catalytic activity. Lapatinib shows a dose-dependent inhibition of EGFR with a $K_i = 3$ nM, HER2 with a $K_i = 13$ nM, and HER4 with a $K_i = 347$ nM (Wood et al., 2004). In a dose response experiment, pre-treatment of MCF-7 cells with 50 nM lapatinib effectively inhibited HRG-induced HER3 phosphorylation and association with MTK1 (Figure 7). Since HER4 has a $K_i$ of 347 nM for lapatinib, EGFR and HER2 were the more likely candidates for lapatinib blockade of HER3 phosphorylation and subsequent MTK1/HER3 association. EGFR is expressed in MCF-7 and T-47D cells and can form heterodimers with HER3 (Eccles, 2011; Yarden et al., 1996). However, we have performed experiments comparing EGF and HRG and found that the EGFR is not activated in response to HRG (data not shown). Together, our results suggest that lapatinib inhibited HER2 and effectively inhibited HER3 phosphorylation and formation of the MTK1/HER3 heterodimer. Additionally in HER2 negative and HER3 positive MDA-MB-231 cells, HRG does not induce formation of the MTK1/HER3 dimer (Figure 7C). Therefore, these data support that HER2 protein expression and kinase activity is required for HER3 phosphorylation and MTK1/HER3 association.
Cell migration requires actin polymerization and intracellular coordination of actin binding proteins, which are regulated by signal transduction and subsequent second messenger signaling (Feldner and Brandt, 2002). Migration is also an important part of cancer cell invasion and metastasis (Yamazaki et al., 2005). HER2 is linked to actin cytoskeletal reorganization in cancer (Feldner and Brandt, 2002) and heregulin stimulation of breast cancer cells enhances the conversion of g-actin to f-actin increasing cell migration (Adam et al., 1998; Hijazi et al., 2000), implicating HER3 with actin cytoskeletal reorganization. Additionally, our studies reveal that MEKK4 interacts with actin through a 40 amino acid region referred to as the actin interacting region (AIR), which is also found in yeast Ssk2p (Yuzyuk et al., 2002b). Deletion of the AIR within MEKK4 impairs the constitutive association between MEKK4 and actin (Figure 8B). These results demonstrate a proximal localization of MEKK4 with the actin cytoskeleton. When MCF-7 cells were pre-treated with cytochalasin D to prevent actin polymerization, the interaction between MTK1 and HER3 was reduced by ~60%, indicating a cytoskeletal connection involving actin linking MTK1 and HER3. Future studies will be needed to determine whether the MTK1/HER3 dimer remains at the plasma membrane or mobilizes via actin filaments to other intracellular locations.

Although others have reported that HRG induces a proliferative response (Yang et al., 2008), we were unable to detect HRG-dependent proliferation in either T-47D (data not shown) or MCF-7 cells (Figure 9A), although we detected an EGF-dependent proliferative response. As the cells were incubated with either HRG or EGF, we noticed
that the cell media developed a more orange color in the presence of HRG. When the pH was recorded, we consistently observed that HRG caused an acidification of the extracellular medium while EGF had no effect. Given that HRG caused an association between MTK1 and HER3, we used siRNA to decrease the expression of MTK1 to determine whether MTK1 was involved in regulating extracellular pH. Although siRNA typically diminished MTK1 expression by only ~60% in MCF-7 cells (see Figure 10D for a representative immunoblot), the change in extracellular pH was always diminished in the absence of MTK1 (Figure 9B, lanes a & c). These results demonstrate that EGF and HRG induce different responses in MCF-7 cells and that the EGF-dependent proliferative response does not correlate with acidification of the extracellular medium. Since HRG did not induce proliferation of MCF-7 cells, we investigated whether HRG induced cell migration by using a scratch assay. After 24 hours of HRG exposure in the presence of NS siRNA, 60% of the scratch area was filled in by cells migrating back into the scratched region (Figure 10A). In contrast, only 20% of the scratch area was filled in with cells migrating back into the scratch region after 24 hours in cells with MTK1 knockdown. Additionally, cells pre-treated with lapatinib migrated back into the scratch area by only 27%. These results demonstrate that HRG induces migration and not proliferation of MCF-7 cells, consistent with the results of Arsani et al. (Asrani et al., 2013). We also demonstrate that MTK1 is required to acidify the extracellular environment. The acidification of the extracellular environment likely helps optimize the enzymatic activity of matrix metalloproteinases or other enzymes needed for the migratory response.
MTK1 is not tyrosine phosphorylated in response to HRG stimulation of T-47D cells (Figure 4A, lane a) or MCF-7 cells (Figure 4A, lane g). However, the association between MTK1 and HER3 is dependent on HRG. Since MTK1 is not tyrosine phosphorylated, it is unlikely that MTK1 is a substrate for HER2. Conversely, we have not investigated whether the catalytic activity of MTK1 is required for the association between MTK1 and HER3. Our data demonstrate phosphorylation of serine 686 of HER3 (LERGEpSIEP) in response to HRG by sequencing using LC-MS/MS (Figure 4B). Since we were unable to sequence any HER3 peptides due to lack of HER3 association with MTK1 in the absence of HRG stimulation, we cannot state whether serine 686 is phosphorylated under basal conditions. Phosphorylation of HER3 at serine 686 has been previously reported, but the functional significance of this modification is not known (Daub et al., 2008). Earlier studies have shown MEKK4 functions as a serine/threonine kinase regulating MKK6, which is upstream of p38 (Gerwins et al., 1997; Mita et al., 2002; Takekawa et al., 1997; Takekawa and Saito, 1998). Our current results suggest that MTK1 functions as a scaffolding protein for phosphoHER3. However, since MTK1 is a serine/threonine kinase, it is possible that the catalytic activity of MTK1 is required to regulate changes in extracellular pH and cell migration.

In summary, HER2/HER3 heterodimerization has been well characterized, but we show for the first time that HRG stimulation results in the formation of a MTK1/HER3
heterodimer, which is sustained for up to 150 minutes in breast cancer cells. We also demonstrate that HER2 kinase activity is needed for association of MTK1 with phosphoHER3. Furthermore, disruption of actin polymerization inhibits HRG induced MTK1/HER3 association, which suggests that f-actin is required to bring MTK1 and phosphoHER3 together linking MTK1 to the actin cytoskeleton. Additionally, we report that MTK1 is required for HRG induced extracellular acidification and cell migration. Lapatinib also inhibited HRG induced extracellular acidification and cell migration, showing that HER2 is required in this signaling process. In conclusion, over-expression of HER2 in breast cancer has been targeted with drugs like trastuzumab and lapatinib, however drug resistance or unresponsiveness persists among many patients (Garrett and Arteaga, 2011; Nahta and Esteva, 2006). The link between HER3 up-regulation to HER2 drug resistance makes HER3 a desirable molecular target for anti-cancer drug development. However, the lack of HER3 catalytic activity has made HER3 somewhat less tractable as a drug target. By identifying the recruitment of MTK1 to phosphoHER3, we now shed new light onto the HER3 branch of the HER2/HER3 signaling tree.
CHAPTER 3

THE ROLE OF MTK1 IN THE GIT1-ASSOCIATED ERK1/2 MAPK PATHWAY IN RESPONSE TO HEREGULIN IN BREAST CANCER CELLS

3.1 INTRODUCTION

Human MAP3K4 (MTK1) functions upstream of mitogen activated protein kinases (MAPKs). Previously, we reported that HRG stimulation of MCF-7 human epithelial breast cancer cells leads to extracellular acidification and cell migration, independent of proliferation. Additionally, we identified tyrosine phosphorylated HER3 associating with MTK1 in response to HRG stimulation that was dependent on HER2 activity. HER2 did not associate with the MTK1/HER3 heterodimer, suggesting HRG dependent HER2 phosphorylation of HER3 occurs transiently. Furthermore, we also reported that MTK1 is required for HRG induced extracellular acidification and cell migration in MCF-7 breast cancer cells. In this study, we further characterized HRG induced association of MTK1 with activated ERK1/2, GIT1, Shc and Grb2 in T-47D and MCF-7 breast cancer cells.
MTK1 has a role in the ERK1/2 MAPK signaling pathway in response to heregulin (HRG) stimulation in T-47D and MCF-7 breast cancer cells. ERK1/2 kinase activity is involved in aberrant signaling that leads breast cancer progression. In addition to MTK1, Shc, Grb2 and GIT1 proteins are all involved in the ERK1/2 MAPK pathway in response to growth factor stimulation. Here we show a novel constitutive association of MTK1 with ERK1/2, GIT1, Shc and Grb2. We also demonstrate that tyrosine-phosphorylated Shc and Grb2 are recruited to MTK1 in response to HRG stimulation. Furthermore, we show that GIT1 is rapidly recruited to MTK1 while activated ERK1/2 is detected with MTK1 after 5 minutes in response to HRG stimulation. Activated ERK1/2 association with MTK1 is sustained up to 150 minutes post HRG stimulation suggesting MTK1 has a role in ERK1/2 activity. MTK1 displays scaffolding properties in the HRG mediated activation of ERK1/2 that may be required for ERK1/2 activity. Identification of the exact role MTK1 plays in HRG mediated ERK1/2 activation may be the key to blocking ERK1/2 mediated breast cancer progression.

Growth factor stimulation results in activation of the Raf, MEK, ERK kinase cascade (Roberts and Der, 2007; Rozakis-Adcock et al., 1992). The activation of ERK1/2 is required for normal cellular processes, such as proliferation, differentiation and migration (Keshet and Seger, 2010; Roberts and Der, 2007). As previously mentioned, growth factors and RTK receptors that regulate ERK1/2 are often over-expressed in breast cancer cells (Kraus et al., 1989; Lemmon, 2003; Muller et al., 1988; Yarden and Sliwkowski, 2001), leading to unregulated ERK1/2 activity.
Shc proteins regulate ERK1/2 activity. There are three characterized Shc proteins, isoforms of 46, 52 and 66 kDa. Shc is tyrosine phosphorylated by activated RTKs in response to growth factor stimulation (Basu et al., 1994; Rozakis-Adcock et al., 1992). Once tyrosine phosphorylated, Shc forms a complex with the adapter protein Grb2, which leads to the activation of SOS and the Ras signaling pathway, resulting in activation of ERK1/2 (Egan et al., 1993; Kumar et al., 1995; Rozakis-Adcock et al., 1992; Skolnik et al., 1993). The Shc proteins have three primary domains, an amino-terminal PTB domain that associates with tyrosine-phosphorylated NPXY motifs, an CH1 domain that is tyrosine phosphorylated, and a SH2 domain that associates with phosphotyrosine residues (Pelicci et al., 1996). Additionally, Shc associates with f-actin through the amino-terminal domain of Shc (Thomas et al., 1995). These three domains allow Shc to associate with other proteins under specific post-translational modifications and regulate their activity in various signaling pathways within the cell.

When a tyrosine kinase receptor becomes activated, via tyrosine phosphorylation, Shc can associate with the receptor either through its PTB. For example, Shc associates with the EGFR, insulin receptor and HER3 proteins when they are phosphorylated on NPXY motifs (Farooq et al., 1999). The HER3 and Shc association is mediated tyrosine 1,329 phosphorylation of the HER3 NPXY motif (Farooq et al., 1999). HER3 is not catalytically active and therefore requires heterodimerization of other HER proteins for
Shc tyrosine phosphorylation. EGF stimulation of the EGFR leads to the tyrosine phosphorylation of Shc (Basu et al., 1994; Rozakis-Adcock et al., 1992). Once tyrosine phosphorylated, Shc is able to form a complex with the adapter protein Grb2. Tyrosine phosphorylation of Shc is required for association of Grb2 and subsequent activation of the Ras ERK1/2 signaling pathway that leads to proliferation of human breast cancer cells (Clark et al., 1996).

GIT1 associates with ERK1/2 in response to growth factors (Yin et al., 2005; Zhang et al., 2010b). GIT1 consists of multiple domains that function as recruitment sites for other proteins. GIT1 has an amino terminal ARF-GTP domain followed by three ankyrin (ANK) repeats, an internal Spa2 homology (SHD) domain, coiled-coiled (CC2) domain and a carboxyl terminal paxillin binding site PBS domain (Hoefen and Berk, 2006). GIT1 acts as a scaffold protein that is required for activation of the MEK1-ERK1/2 pathway in response to angiotensin II (AngII) and EGF stimulation (Yin et al., 2004). GIT1 is also tyrosine phosphorylated and regulated by the c-Src non-receptor tyrosine kinase in response to agonist stimulation (Hoefen and Berk, 2006; Sato et al., 2008).

Proteins that function in the MTK1 pathway have not been fully characterized nor has the regulation of MTK1 kinase activity. Previously we have shown mouse MAP3K4 (MEKK4) to be regulated through activation of cytokine receptor [i.e., IFNγ, (Halfter et al., 2005)] and GPCR [AngII, (Derbyshire et al., 2005)]. In this study we investigated
whether MTK1 is also regulated by the activation of RTK in MCF-7 and T-47D epithelial breast cancer cells. We identified constitutive association of MTK1 with ERK1/2, p52 Shc, Grb2 and GIT1. Additionally, we identified an increased association of MTK1 with activated ERK1/2, tyrosine phosphorylated Shc, Grb2, and GIT1 in response to HRG stimulation. We demonstrate a novel relationship between MTK1 and the ERK1/2 MAPK signaling pathway in response to HRG stimulation in MCF-7 and T-47D breast cancer cells.
3.2 MATERIALS AND METHODS

3.2.1 CELL CULTURE AND TREATMENTS

T-47D cells were cultured in Dulbecco’s modified Eagles medium high glucose (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Prior to experimental procedures, T-47D cells were cultured in DMEM supplemented only with 1% penicillin-streptomycin for 16 hrs. Cells were stimulated with 10 nM heregulin-β3 (HRG) EGF-Domain (Millipore Cat # 01-201) for 12 minutes unless otherwise indicated or with 0.3 M sorbitol for 30 minutes. Pre-treatment with 50 µM PD98059 MEK1/2 inhibitor was performed for 1 hr prior to HRG stimulation where indicated.

3.2.2 WESTERN BLOTTING AND ANTIBODIES

Cells were lysed in MAPK lysis buffer (70 mM β-glycerol phosphate, 1 mM EGTA, 1 mM dithiothreitol, 2 mM MgCl₂ and 0.5% Triton X-100) with protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride, 127.4 KIU/ml aprotinin (Calbiochem Cat # 616399), 10 µM leupeptin and with 0.5 mM sodium orthovanadate (a tyrosine phosphatase inhibitor). Proteins were resolved by 5% – 12.5% gradient SDS-PAGE gels and transferred onto Protran 0.45 µm nitrocellulose blotting membrane (BioExpress Cat # F-3120-7). Membranes were blocked with 5% non-fat dry milk in 25 mM Tris-HCl, pH
7.4, 137 mM NaCl, 2.7 mM KCl and 0.15% Tween 20 (TBS-T). Immunostaining was performed in 5% non-fat dry milk in TBS-T and detected using chemiluminescence reagent (100 mM Tris pH 8.5, 250 mM luminol, 92 mM p-coumaric acid and 0.018% H$_2$O$_2$). Images were obtained using ChemiDoc™ XRS+ (BIO-RAD) and quantification was performed with Image Lab Software. Antibodies were purchased from BD Biosciences (Grb2, Cat # 610112; Shc Cat # 610879), Cell Signaling (ERK1/2 #4696; phosho-ERK1/2 #9106; MEK1/2 #9122; phospho-MEK1/2 #9121; p38 #9212; phospho-p38 #9212; anti-mouse HRP-conjugated #7076S; anti-rabbit HRP-conjugated #7074S), Millipore (phosphotyrosine Clone 4G10 #05-321), Santa Cruz Biotechnology Inc. (GIT1 sc-135925) and MTK1 antibodies were developed as we previously described (Derbyshire et al., 2005).

3.2.3 COUPLING OF MTK1-P ANTIBODY TO PROTEIN-A SEPHAROSE BEADS

To couple MTK1-P rabbit polyclonal antibody, 55 µg of IgG antibody was combined per 100 µl Protein-A Sepharose beads plus 500 µl of PBST (phosphate buffered saline with 0.1% Tween 20) (a total of 825 µg of IgG antibody, 1.5 ml Protein-A Sepharose beads and 7.5 ml PBST) and tumbled for 1 hr at room temperature. The beads were then washed 3 times with 10 ml of PBST. Next the beads were washed 3 times with 10 ml of 0.2 M sodium borate. Coupling was completed by adding 13 ml of 22 mM DMP.
(dimethyl pimelimidate Pierce Cat# 21667) in 0.2 M sodium borate to the antibody Protein-A Sepharose beads and tumbling for 1 hr at room temperature. The beads were washed with 0.2 M ethanolamine in 0.2 M sodium chloride for 2 hours at room temperature. To remove any non-coupled MTK1-P IgG antibody, the beads were pre-eluted with 10 ml of 0.1 M glycine in 0.1 M sodium chloride and washed in 10 ml of 0.5 M Hepes pH 7.4 (repeated 3 times). Coupled beads were then washed 2 times with 0.2 M ethanolamine followed by 2 washes with PBS (phosphate buffered saline) pH 7.4. MTK1-P antibody coupled Protein-A Sepharose beads were then re-suspended in an equal volume of PBS and stored at 4°C. The beads were then used in immunoprecipitation experiments followed by trypsin digestion, immobilized metal ion affinity chromatography (IMAC) enrichment and Orbitrap LC-MS analysis to identify proteins that associate with MTK1 during HRG stimulation.

3.2.4 IMMUNOPRECIPITATION EXPERIMENTS

T-47D cells were stimulated with 10 nM HRG, 0.3 M sorbitol or vehicle. The cells were then lysed in MAPK lysis buffer and 2 mg of cell extract was immunoprecipitated for 1 hr at 4°C with rabbit anti-MTK1 polyclonal antibodies (Derbyshire et al., 2005); or rabbit anti-GIT1 polyclonal antibody (Cell Signaling #2919). Immune complexes recovered using protein A-Sepharose beads (Sigma Cat# P3391) were washed twice with ice cold MAPK lysis buffer and denatured with Laemmli sample buffer. Proteins were separated
by 5% – 12.5% gradient SDS-PAGE gels and transferred to nitrocellulose membranes that were then subjected to immunoblot analysis as indicated.

### 3.2.5 GST-GRB2 FUSION PROTEIN PULL-DOWNS

T-47D cells were stimulated with 10 nM HRG or vehicle for 12 minutes. The cells were washed with ice cold PBS and lysed in MAPK lysis buffer, 2 mg of cell extract was then incubated with 5 µg of fusion protein for 1 hr. GST-Grb2 fusion proteins (GST-Grb2 full length, GST-Grb2 SH2 domain, GST-Grb2 amino-terminal SH3 domain (SH3-N), and the GST-Grb2 carboxyl-terminal SH3 domain (SH3-C) were generously provided by A. Shaw from the University of Washington, CO. Missouri. Pull-down complexes recovered using glutathione Sepharose 4B (Calbiochem Cat# 17-0756-01) were washed twice with ice cold MAPK lysis buffer. Proteins were separated by SDS-PAGE gels and transferred to nitrocellulose membranes as described above.
3.2.6 *siRNA KNOCKDOWN EXPERIMENTS*

MCF-7 cells were seeded (onto 10 cm cell culture plates, VWR # 353003) at a density of 5.0 x 10^6 cells and allowed to adhere for 16 hours. The cells were then transfected with 434 pmol of siRNA specific for GIT1 (Thermo Scientific ON-TARGETplus SMARTpool, Human GIT1 Cat # L-020565-00-0005) or non-targeting (NS) (ON-TARGETplus Non-targeting Pool Cat # D-001810-10-05) as control using lipofectamine 2000 according to manufacture recommendations. The cells were transfected with siRNA a second time 24 hours later using lipofectamine 2000 to enhance knockdown efficiency. Twenty four hours after the second transfection the cells were detached using 0.25% trypsin (Invitrogen # 15050-065). The cells were counted and seeded onto 10 cm culture dishes (VWR # 62406-165) at a density of 7.0 x 10^6 cells per dish. After 16 hours, the cells were serum starved for four hours followed by stimulation with HRG for 12 minutes. GIT1 knockdown efficiency was determined by immunoblot analysis using 150 µg whole cell lysate. Each condition was performed in triplicate and repeated a minimum of 3 times. Data shown for each condition represents n = 3. Statistical analysis was performed as described above.
3.3 RESULTS

3.3.1 ASSOCIATION OF TYROSINE PHOSPHORYLATED PROTEINS WITH MTK1

To further investigate RTK regulation of MTK1 we selected MCF-7 and T-47D cells which express moderate levels of the HER1-4 proteins (Beerli and Hynes, 1996; Carraway et al., 1994). HRG regulates HER3 and HER4, allowing dimerization with each other or dimerization with HER1 and HER2 proteins (Aguilar et al., 1999; Carraway and Cantley, 1994; Neve et al., 2002). MCF-7 or T-47D cells were stimulated with HRG or sorbitol to induce osmotic stress and activate MTK1, since MTK1 is known to function in the p38 MAP Kinase pathway (Abell et al., 2007; Aissouni et al., 2005; Takekawa and Saito, 1998). After stimulation, MTK1 was immunoprecipitated utilizing two different polyclonal rabbit antibodies directed against two different epitopes of MTK1. The MTK1-P antibody is directed against an amino terminal epitope of MTK1 that is proline rich and the MTK1-A antibody is directed against an internal epitope of MTK1 that is alanine rich (Figure 11).
FIGURE 11. MTK1 IMMUNOPRECIPITATION OF T-47D BREAST CANCER CELLS STIMULATED BY HRG AND SORBITOL.

A schematic representation of MTK1 domains identifying antibody epitopes directed against MTK1. The MTK1 (P) antibody recognizes an amino terminal region (amino acids 18–139) of MTK1 that is proline rich, while the MTK1 (A) antibody recognizes an internal region (amino acids 1,102–1,255) of MTK1 that is alanine rich (Panel A). Cells were grown to subconfluence, serum starved for 16 hours and stimulated with 10 nM HRG for 12 minutes or 0.3 M sorbitol for 30 minutes. Lysates were incubated with anti MTK1 (A) or anti MTK1 (P) antibody and precipitated with Protein A–Sepharose. Immunoprecipitated proteins were separated by 5-12.5% gradient SDS-PAGE. Proteins were transferred to nitrocellulose membrane and immunoblotted using the indicated antibodies. The membrane showing the 52 kDa tyrosine phosphorylated protein was stripped and re-probed with Shc antibody (Panel B).
Immunoprecipitated MTK1 and associated proteins were resolved using SDS-PAGE, followed by transfer to nitrocellulose membrane. Tyrosine phosphorylated proteins were identified by immunoblotting with a monoclonal mouse antibody (clone 4G10) that specifically recognizes phosphotyrosine residues. When using the MTK1-P antibody for immunoprecipitation, two unique tyrosine phosphorylated proteins co-immunoprecipitated with MTK1 in response to HRG stimulation (Figure 11B, lane a). One protein associating in this complex has a molecular weight of 84 kDa, but is also tyrosine phosphorylated in basal conditions and in response to sorbitol. However, HRG and sorbitol stimulation consistently showed stronger tyrosine phosphorylation of the 84 kDa protein compared to vehicle stimulated cells (Figure 11B, lanes a-c). The second protein associating in this complex has a molecular weight of 52 kDa and consistently displayed strong tyrosine phosphorylation in response to HRG stimulation, but was not phosphorylated in vehicle and sorbitol stimulated cells (Figure 11B, lanes a-c). In contrast, when using our MTK1-A antibody to immunoprecipitate MTK1, the 84 kDa protein was less detectable and the 52 kDa tyrosine phosphorylated protein showed a consistently weaker signal (Figure 11B compare lanes a and d) even though similar levels of MTK1 were immunoprecipitated with either antibody (FIG 11B, lanes a-f). These data reveal the importance of using multiple antibodies directed against different epitopes when studying protein signaling. If we only utilized the MTK1-A antibody in the MTK1 immunoprecipitations, the proteins identified with the MTK-P immunoprecipitations could have been overlooked due to weaker signals.
3.3.2 ASSOCIATION OF SHC WITH MTK1

The immunoprecipitation experiments using the MTK1-P and MTK1-A antibodies followed by Western blotting with anti-phosphotyrosine antibody demonstrate that MTK1 is regulated by HRG. The next experiments were designed to identify the 84 and 52 kDa proteins. Since HRG is known to induce proliferation (Ethier et al., 1996; Jones et al., 1996; Lim et al., 2009; Yang et al., 2008), we hypothesized that the 52 kDa protein might be Shc. To test this hypothesis, the same nitrocellulose membrane that was used for immunoblotting against phosphotyrosine was stripped and re-immunoblotted with antibody specific for the 46, 52 and 66 kDa isoforms of Shc.

Stimulation of T-47D cells with HRG caused tyrosine phosphorylation of the 52 kDa isoform of Shc and a HRG dependent increased association of MTK1 with Shc compared to vehicle and sorbitol stimulation (Figure 11B, lanes a-c). Furthermore, tyrosine phosphorylated Shc was observed associating with MTK1 in MCF-7 cells (data not shown). The membrane was also immunoblotted for the adapter protein Grb2, since tyrosine phosphorylated Shc has been shown to associate with Grb2 (Rozakis-Adcock et al., 1992; Skolnik et al., 1993). The association between Grb2 and MTK1 increased in a HRG dependent manner compared to vehicle and sorbitol stimulation (FIG 11B, lanes a-c). The increase in association of Grb2 with MTK1 paralleled the increase in association of Shc with MTK1. The fact that Grb2 was present in this complex of proteins and it is
known to associate with tyrosine phosphorylated Shc (Rozakis-Adcock et al., 1992; Skolnik et al., 1993) gave us further confidence that we had successfully identified the 52 kDa tyrosine phosphorylated protein associating with MTK1 under HRG stimulation as 52 kDa Shc. Increased association of 46 kDa Shc isoform was also observed with the MTK1-P immunoprecipitation during HRG stimulation, however the increase was not as dramatic as the 52 kDa isoform of Shc. The 66 kDa Shc isoform was weakly detected in the MTK1-P immunoprecipitation, however it was not tyrosine phosphorylated (data not shown).

3.3.3 MTK1 ASSOCIATES WITH GIT1

The University of Arizona Southwest Environmental Health Sciences Center (SWEHSC) proteomics core facility was utilized to identify HRG dependent tyrosine phosphorylated proteins associated with MTK1. To increase the probability of identifying these proteins six HRG stimulated immunoprecipitations were pooled together, and as a control six vehicle stimulated immunoprecipitations were pooled together, where each immunoprecipitation started with 2.0 milligrams of cell extract. Instead of resolving these proteins and excising them from a polyacrylamide gel for peptide digestion, an in solution preparation of precipitated proteins was used, to allow for identification of multiple proteins in one step. The presence of MTK1-P antibody IgG heavy chain in the immunoprecipitations would create significant background noise with the analysis of
digested peptides. To eliminate IgG heavy chain in the digestion procedures, the MTK1-P antibody was covalently coupled to Protein-A Sepharose beads using dimethyl pimelimidate (DMP). After immunoprecipitations the samples were pooled together and loaded onto a BIO-RAD disposable chromatography filter column (Cat # 732-6008). The columns were washed six times with 1X TBS. Proteins were then eluted with 0.1 M acetic acid, leaving MTK1-P IgG heavy chain attached to Protein-A Sepharose beads on the chromatography filter column. Acetic acid was removed via vacuum centrifugation and protein samples were digested into peptides with trypsin. The samples were then enriched for phosphopeptides using an immobilized metal ion affinity chromatography (IMAC), both the flow through and enriched samples were analyzed via Orbitrap LC-MS.

As an internal control, MTK1 was positively identified in the phosphopeptide analysis indicating the immunoprecipitations for MTK1 were successful. HRG or sorbitol stimulation of T-47D cells followed by MTK1 immunoprecipitation produced tyrosine phosphorylated protein that associates with MTK1 (Figure 11B, lanes a-c). Phosphopeptide analysis of MTK1-P immunoprecipitated proteins identified GIT1 in the complex of proteins associating with MTK1 in the presence of HRG and vehicle stimulation (Figure 12A & 12B). In the peptide analysis 31.3% and 30.4% of the total protein coverage for GIT1 was identified with HRG and vehicle stimulation respectively, and several peptides had a 99.9% high confidence for positive identification (Figure 12A & 12B). GIT1 has a molecular weight of 84.3 kDa, which was consistent with the tyrosine phosphorylated protein (Figure 11B). In addition, several high confidence GIT1
peptides had numerous post-translational modifications of tyrosine and serine/threonine phosphorylation (Figure 12A & 12B). To further validate positive identification of GIT1 associating with MTK1, T-47D cells were stimulated with HRG and MTK1 was immunoprecipitated with MTK-P antibody, as described above. Proteins were resolved by SDS-PAGE and immunoblot analysis was performed for GIT1, and then the membrane was stripped and immunoblotted for phosphotyrosine (Figure 12C). GIT1 displayed an increased association with MTK1 in response to HRG stimulation compared to vehicle stimulated cells and the tyrosine phosphorylated protein coincided with GIT1 (Figure 12C). This result provided further evidence that we had positively identified GIT1 as the 84 kDa tyrosine phosphorylated protein associating with MTK1. The MTK1/GIT1 association was also observed in MCF-7 breast cancer cells (data not shown). In summary, proteomics and immunoblot analysis of MTK1-P immunoprecipitations after HRG stimulation show an association of MTK1 and GIT1. Although there appears to be a constitutive association, GIT1 tyrosine phosphorylation and association with MTK1 increases with HRG stimulation.
## GIT1 Peptides Associated with MTK1 after HRG Stimulation 31.3% Gene coverage

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High ≥ 99.9%, Medium ≥ 95.0%, Low ≤ 94.0%
 GIT1 Peptides Associated with MTK1 after Vehicle Stimulation 30.4 % Gene coverage

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FIGURE 12. IMMUNOBLOTING OF GIT1 IN THE MTK1 IMMUNOPRECIPITATION.

To identify the tyrosine phosphorylated 84 kDa protein in the MTK1 immunoprecipitations, MTK1 was immunoprecipitated from T-47D cells stimulated for 12 minutes with 10 nM HRG or vehicle. LC-MS analysis from the MTK1 immunoprecipitations positively identified tyrosine phosphorylated peptides of GIT1 (Panels A & B). To further assess the results obtained from the mass spectrometry analysis, MTK1 was immunoprecipitated from T-47D cells stimulated for 12 minutes with 10 nM HRG or vehicle. Immunoprecipitated proteins were separated by 5-12.5% gradient SDS-PAGE. Proteins were transferred to nitrocellulose membrane and immunoblotted using the indicated antibodies. The membrane showing the 84 kDa GIT1 protein was stripped and re-probed with phosphotyrosine antibody (Panel C).
3.3.4  ERK1/2 ASSOCIATES WITH MTK1 AND IS ACTIVATED IN RESPONSE TO HRG STIMULATION

The association of Shc, Grb2 and GIT1 with MTK1 strongly suggested that MTK1 signals to the ERK MAPK kinase pathway. Since MTK1 serves as a scaffold for GIT1 and GIT1 recruits MEK1 for ERK activation (Stockton et al., 2007; Yin et al., 2004; Zhang et al., 2010b), we wanted to determine whether ERK1/2 were recruited to MTK1 in response to HRG. T-47D cells were stimulated with HRG from 30 seconds to 20 minutes and MTK1 was immunoprecipitated with the MTK1-P antibody. Non-activated ERK1/2 were constitutively associated with MTK1 (Figure 13, lanes a-k), while activated ERK1/2 were detected with MTK1 five minutes after stimulation (Figure 13, lanes e-k). Not only were activated ERK1/2 detected with MTK1 after five minutes using phospho-ERK1/2 antibody, but phosphorylated ERK1/2 were detected due to retardation of mobility by SDS-PAGE (Figure 13, lanes e-k, bottom panel). The HRG dependent association of activated ERK1/2 with MTK1 was also observed in MCF-7 cells (data not shown). In addition, GIT1 recruitment rapidly increased at 30 seconds and remained elevated up to 10 minutes after HRG stimulation (Figure 13, lanes b-g). GIT1 association then decreased back to basal levels after 12.5 minutes (Figure 13, lane h).
FIGURE 13. GIT1 IMMUNOPRECIPITATION WITH MTK1.

GIT1 immunoprecipitation with MTK1 increased after 30 seconds of HRG stimulation and activated ERK1/2 immunoprecipitated with MTK1 after 5 minutes. Re-probe of GIT1 immunoblot showed GIT1 to be tyrosine phosphorylated. Immunoblot analysis for phospho-ERK1/2 revealed activated ERK1/2 to immunoprecipitate with MTK1 after 5 minutes of HRG stimulation and re-immunoblot demonstrated ERK1/2 to be constitutively associated with MTK1 immunoprecipitations.
In an extended time course (0-150 minutes) of HRG stimulation, activated ERK1/2 remained associated with MTK1 for up to 150 minutes (Figure 14A, lanes b-j). Similarly, the recruitment of tyrosine phosphorylated Shc with MTK1 began after 5 minutes and was sustained up to 150 minutes. Grb2 displayed a related pattern of association with MTK1 in response to HRG (Figure 14A, lanes b-j). Whole cell extracts of T-47D cells demonstrated that ERK1/2 activation begins after 5 minutes, in response to HRG, and is sustained up to 150 minutes (Figure 14B lanes b-j). In contrast, p38 activation begins after 5 minutes in response to HRG and peaks after 10 to 15 minutes, demonstrating that sustained MAP kinase activity in response to HRG stimulation is unique to the ERK1/2 MAP kinases (Figure 14B).
FIGURE 14. MTK1 ASSOCIATES WITH SUSTAINED ACTIVATED ERK1/2.

Immunoprecipitation of MTK1 was performed and immunoblot analysis for phospho-ERK1/2 revealed activated ERK1/2 to immunoprecipitate with MTK1 after 5 minutes and up to 150 minutes post HRG stimulation (Panel A). Tyrosine phosphorylated Shc was also shown to increase in association with MTK1 immunoprecipitation in response to HRG after 5 minutes and up to 150 minutes (Panel A). Whole cell lysates revealed ERK1/2 to be activated after 5 minutes and sustained up to 150 minutes post HRG stimulation, while phospho-p38 activation appeared to only be transient (Panel B).
These data demonstrate that GIT1 and ERK1/2 are constitutively associated with MTK1. Moreover, GIT1 recruitment is rapid, happening within 30 seconds of HRG stimulation and overlapping with ERK1/2 activation. However, it is interesting to note that tyrosine phosphorylation of GIT1 increases slightly in response to HRG but tyrosine phosphorylation of GIT1 does not coincide with increased recruitment of GIT1 and MTK1. Further investigation into GIT1 tyrosine phosphorylation sites will be required in order to delineate the relationship between GIT1 and MTK1.

The rapid recruitment of GIT1 to MTK1 followed by ERK1/2 activation in response to HRG, suggests that GIT1 could be recruiting MEK to this complex to activate ERK1/2. Stimulation of MCF-7 or T-47D cells with HRG elicits the activation of ERK1/2 that was associated with MTK1 suggesting that HRG activates MEK1/2. However, MEK1/2 was not detected with immunoprecipitated MTK1, likely due to transient association (data not shown). To address whether MEK1/2 activates ERK1/2 that was associated with MTK1, T-47D cells were pre-treated with PD98059, a MEK1/2 inhibitor. PD98059 completely blocked HRG dependent activation of ERK1/2 that was associated with MTK1 (Figure 15A, lanes f-j). Pre-treatment of cells with PD98059 inhibited HRG stimulated activation of both MEK1/2 and ERK1/2 (Figure 15B, lanes f-j). These data demonstrate HRG stimulation of T-47D cells leads to increased recruitment of GIT1 to MTK1 followed by activation of ERK1/2 that is also associated with MTK1. These data also show that MEK1/2 is required for the activation of ERK1/2 that is associated with MTK1.
Figure 15

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5x

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pMEK1/2

MEK1/2

Re-probe

kDa

50

50

50

a b c d e f g h i j

pERK1/2

ERK1/2

Re-probe

Lysates

MTK1 IP
FIGURE 15. MEK1/2 ACTIVITY IS REQUIRED FOR THE ACTIVATION ERK1/2 THAT IS ASSOCIATED WITH MTK1.

To determine whether MEK1/2 was required for the activation of ERK1/2 that was associated with MTK1, T-47D cells were pre-treated with PD98059 (a MEK1/2 inhibitor) prior to HRG stimulation. Immunoprecipitation of MTK1 showed that pre-treatment with PD98059 completely blocked the activation of ERK1/2 that was associated with MTK1 (Panel A). Whole cell lysates showed that pre-treatment with PD98059 blocked the activation of MEK1/2 and subsequently the activation of ERK1/2 (Panel B).
3.3.5 GIT1 ASSOCIATES WITH GRB2

Grb2 has an internal SH2 domain that is flanked by two SH3 domains (Matuoka et al., 1992; Yuzawa et al., 2001). SH2 domains associate with tyrosine phosphorylated residues, while SH3 domains associate with proline rich regions. Since Grb2 is an adapter protein, the association between Grb2 and MTK1 could be mediated by either SH2 or SH3 domains. However, MTK1 is not tyrosine phosphorylated in the presence of HRG stimulation (Sollome et al., 2013), but MTK1 does have an amino-terminal proline-rich region (Figure 11A). Therefore, Grb2 could associate directly with MTK1 via one or both SH3 domains. The multiple domains of GIT1 function as recruitment sites for proteins, therefore GIT1 may also interact directly with MTK1. To determine the association status of Grb2 and GIT1 with MTK1 the following experiments were performed. GST-Grb2 fusion proteins consisting of full length Grb2, the SH2 domain, the amino-terminal SH3 domain (SH3-N), or the carboxyl-terminal SH3 domain (SH3-C) were utilized to perform pull-down experiments (Figure 16A). T-47D cells were stimulated with HRG or vehicle, and then 2 mg of cell extracts were combined with GST-Grb2 fusion proteins bound to Glutathione Sepharose 4B beads. GIT1 associated with full length Grb2, and the SH3-N domain (Figure 16B, lanes b-c and f-g). There was no detectable association of GIT1 with GST-Grb2 SH2 or SH3-C domains (Figure 16B, lanes d-e and h-j). It is significant to note that GIT1 displayed a strong association with GST-Grb2 SH3-N fusion protein (Figure 16B, lanes f and g). These data suggest GIT1 and Grb2 association is mediated through the amino-terminal SH3 domain of Grb2. Shc
association with Grb2 was observed in all Grb2 pull-downs and increased with HRG stimulation. Immunoprecipitation of MTK1 was also performed as a control (Figure 16B, lane a). MTK1 was not detected in the GST-Grb2 pull-downs, suggesting that MTK1 association with Grb2 is indirect (Figure 16B, lanes b-j). To further validate the GIT1 and Grb2 association, T-47D cells were stimulated with HRG followed by GIT1 immunoprecipitation. Immunoprecipitation of MTK1 was also performed as a control. Immunoblot analysis revealed both Grb2 and Shc to associate with GIT1 (FIG 16C lanes a-b). These data demonstrate a constitutive association between GIT1, Grb2 and Shc. MTK1 was not detected when GIT1 was immunoprecipitated, suggesting that MTK1 and GIT1 association is indirect or the GIT1 antibody interferes with the MTK1 binding site on GIT1.

3.3.6 GIT1 ASSOCIATES WITH ACTIVATED ERK1/2

Since GIT1 co-localizes with activated ERK1/2 in response to EGF stimulation (Yin et al., 2005; Zhang et al., 2010b), we postulated that activated ERK1/2 also associates with GIT1 during HRG stimulation. To investigate GIT1 and active ERK1/2 association, T-47D cells were stimulated with HRG or vehicle and 2 mg of cell extract was used to immunoprecipitate GIT1. Immunoprecipitation of MTK1 was also performed as a control. Immunoblot analysis using specific antibody for phospho-ERK1/2, revealed activated ERK1/2 to associate with GIT1 in response to HRG stimulation (Figure 16D,
lane a). Not only were ERK1/2 associated with GIT1 as detected by using phospho-ERK1/2 antibody, but phosphorylated ERK1/2 were detected due to retardation of mobility by SDS-PAGE (Figure 16D bottom panel). These data demonstrate constitutive association between GIT1 and ERK1/2 and the association between these two proteins does not require MTK1 (Figure 16D top panel). They also demonstrate that during HRG stimulation phospho-ERK1/2 associates with GIT1.
Figure 16

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GST-Grb2 fusion proteins

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FIGURE 16. GIT1 ASSOCIATES WITH THE GRB2 AMINO-TERMINAL SH3 DOMAIN.

GST-fusion proteins of full length Grb2, the SH2 domain, the amino-terminal SH3 domain (SH3-N), or the carboxyl-terminal SH3 domain (SH3-C) were utilized to perform pull-down experiments (Panel A). Immunoblot analysis revealed GIT1 to pull-down with the amino-terminal SH3 domain of Grb2 (Panel B). GIT1 immunoprecipitation from T-47D lysates also revealed Grb2 to immunoprecipitate with GIT1 (Panel C). GIT1 immunoprecipitation also showed activated ERK1/2 to immunoprecipitate with GIT1 in response to HRG stimulation of T-47D cells (Panel D).
3.4 DISCUSSION

Ectopic expression of MTK1 in Ssk2/Ssk22-deficient yeast results in activation of the p38 MAP kinase pathway, suggesting that MTK1 functions upstream of this pathway in mammalian cells (Takekawa et al., 1997). MTK1 also activates JNK pathways but not ERK in HEK293 cells (Gerwins et al., 1997). In addition, over-expression of dominant-negative MEKK4, which is the mouse homologue of MTK1, blocked differentiation of carcinoma cells via the JNK MAP kinase pathway (Kanungo et al., 2000). These studies demonstrate that over-expression of MTK1 regulates both the p38 and JNK MAP kinase pathways. In this study, we have focused on the signaling properties of endogenous MTK1. We have identified a constitutive association of MTK1 with the p46 and p52 isoforms of Shc, Grb2, GIT1, and ERK1/2. Moreover, we demonstrate a heregulin-dependent recruitment of activated and tyrosine phosphorylated p46 and p52 Shc, although p66 Shc is activated by HRG, it does not associate with MTK1 (data not shown). In addition, with HRG stimulation activated ERK1/2 associates with MTK1. The physical association of MTK1 with Shc and ERK define a novel signaling pathway between the MAP3K, MTK1, and the ERK1/2 MAP kinase pathway that involves GIT1.

GIT1 is an Arf GAP that is recruited to MTK1 in response to heregulin. The GAP activity of GIT1 regulates Arf GTPase activity (Premont et al., 1998) and the interaction with PAK coordinates cytoskeletal reorganization (Kodama et al., 2002). Although the Arfs and Arf GAPs have been reported to control membrane traffic and remodeling of the
actin cytoskeleton (Randazzo et al., 2007), it appears that GIT1 recruits MEK to the MTK1-GIT1-ERK1/2 complex since inhibition of MEK activity with PD98059 prevents activation of MTK1-bound ERK1/2. Our results are consistent with those of Yin et al. who demonstrated MEK recruitment to GIT1 which was required for sustained activation of MEK1 and ERK1/2 after stimulation with AngII or EGF (Yin et al., 2004). Our results now add HRG to the growing list of hormones and growth factors that activate ERK1/2 in a sustained manner via GIT1. The significance of sustained ERK activation in HRG signaling will be further elucidated when MTK1-bound ERK substrates are identified.

We demonstrate that GIT1 interacts with MTK1 although it is not clear how these proteins associate. GIT1 consists of five functional domains that include Arf-GAP, ankyrin repeat, Spa homology domain, coiled-coil, and paxillin binding site (Hoefen and Berk, 2006; Inoue and Randazzo, 2007). In addition to the serine/threonine kinase domain, MTK1 is characterized as having a GADD45 binding domain, an autoinhibitory domain, a dimerization domain (Miyake et al., 2007), and a Cdc42/Rac interactive binding (CRIB) domain (Fanger et al., 1997). The dimerization domain of MTK1 consists of amino acids 982-1012, mediates trans autophosphorylation of MTK1 (Miyake et al., 2007), and is adjacent to the alanine-rich antibody epitope recognized by the MTK1(A) antibody (Figure 11A). The MTK1(A) antibody immunoprecipitates MTK1, but GIT1 weakly co-precipitates with MTK1 using this antibody strongly suggesting that GIT1 and the MTK1(A) antibody compete for the same or adjacent epitope. In contrast,
a GIT1 antibody that recognizes the carboxyl-terminal paxillin binding site of GIT1 does not immunoprecipitate MTK1 (Figure 16C). Based on these data it is tempting to speculate that when GIT1 binds the cytoskeleton through paxillin, then GIT1 does not associate with MTK1. These data indicate that the alanine-rich region of MTK1 and the carboxyl-terminus of GIT1 are involved in mediating the interaction between the two proteins. However, the interaction between MTK1 and GIT1 appears to be indirect since MTK1 was not detected in GIT1 immunoprecipitations (Figure 16 C & D). It is also possible that interaction between MTK1 and GIT1 is mediated by another protein, perhaps a cytoskeletal protein like paxillin.

In response to heregulin, GIT1 recruitment to MTK1 increases approximately 3-fold within 30 seconds of stimulation while the net tyrosine phosphorylation of GIT1 remains essentially unchanged. GIT1 recruitment to MTK1 does not correlate with ERK1/2 activity, which occurs a few minutes later (Figure 13). These results suggest that GIT1 may have another role in the MTK1-ERK1/2 complex. The Arf GAPs have been reported to affect five different cytoskeletal structures that include focal adhesions, invadopodia, podosomes, lamellipodia, and circular dorsal ruffles [reviewed in (Randazzo et al., 2007)]. These cytoskeletal structures are important in normal physiology, but also contribute to cellular behaviors associated with aggressive cancer phenotypes such as invasive cell motility. These phenotypes are especially relevant in tumors that might over-express heregulin and its cognate ErbB receptors. Thus during the early time points of HRG stimulation, the Arf GAP activity, and not the MEK
recruiting activity of GIT1 may be important in cytoskeletal remodeling associated with receptor tyrosine kinase activity and internalization.

Phosphorylation of GIT1 on tyrosine 392 is mediated by Src (Haendeler et al., 2003; Wang et al., 2009). Our first detection of GIT1 was as a tyrosine phosphorylated protein that associates with MTK1 in response to HRG in T-47D breast cancer cells. In those experiments, HRG stimulation was typically for 10-12 minutes and we observed an approximately 2-fold increase in tyrosine phosphorylation of GIT1. Analysis of tryptic peptides derived from MTK1 immunoprecipitations after HRG stimulation demonstrates tyrosine 392 phosphorylation of GIT1. The detected peptides had both high correlation and high confidence scores (Figure 12). Peptides derived from the vehicle stimulated condition demonstrated phosphorylation of tyrosine 392, although the peptides were generally reported as medium to low confidence peptides. A phosphospecific antibody directed against tyrosine 392 would be useful to monitor phosphorylation during the time course of HRG stimulation. In addition to tyrosine phosphorylation, a number of other GIT1 phosphorylated amino acids were detected by mass spectrometry. Although we have not detected MTK1 kinase activity at the early time points of HRG stimulation when using catalytically-inactive recombinant MKK6 (data not shown), it is possible that MTK1 phosphorylates GIT1 in response to heregulin. Future studies will investigate GIT1 phosphorylation at the early time points of HRG stimulation since our current data has only addressed GIT1 phosphorylation after 12 minutes of HRG stimulation. It is also
possible that MTK1 phosphorylates other proteins associated in the MTK1-GIT1-ERK1/2 complex.

Stimulation of T-47D breast cancer cells with HRG is known to activate RTKs leading to the activation of the Ras, Raf, MEK, ERK1/2 signaling cascade (Fiddes et al., 1998). The ERK1/2 MAP kinase cascade is required for normal cellular processes, such as proliferation, differentiation and migration (Keshet and Seger, 2010; Roberts and Der, 2007). Growth factors and the RTK receptors that regulate ERK1/2 are often over-expressed in breast cancer cells (Kraus et al., 1989; Lemmon, 2003; Muller et al., 1988; Yarden and Sliwkowski, 2001), leading to increased ERK1/2 activity. For example, increased ERK1/2 activation in response to growth factor up-regulation plays a critical role in cellular proliferation and migration of oncogenic phenotypes including breast cancer (Aguilar et al., 1999; Deng et al., 2000; Falls, 2003; Fiddes et al., 1998; Gee et al., 2001; Holbro et al., 2003b; Keshamouni et al., 2002; Keshet and Seger, 2010; Meloche and Pouyssegur, 2007; Stove and Bracke, 2004; Tan et al., 2005; Vicent et al., 2004; Xing and Imagawa, 1999; Yang et al., 2008; Zhang et al., 2011). Even though HRG stimulation leads to proliferation of T-47D cells (Fiddes et al., 1998), the signaling mechanisms responsible for the activation of ERK relative to the scaffolding properties of MTK1 have not been reported to date.
In this study, we have identified a constitutive association of MTK1 with p52 Shc, Grb2, GIT1 and ERK1/2 in T-47D breast cancer cells. Shc, Grb2 and GIT1 proteins are all associated with the Ras, Raf, MEK and ERK MAPK signaling pathway (Basu et al., 1994; Bonfini et al., 1996; Yin et al., 2004; Yin et al., 2005; Zhang et al., 2010b). We have identified an increased association between MTK1 with activated ERK1/2, tyrosine phosphorylated Shc, Grb2 and GIT1 in the presence of HRG suggesting that MTK1 is involved in RTK signaling and may have a role in the activation of ERK1/2. The functional consequences of prolonged association of activated ERK1/2 with MTK1 have yet to be determined. It is highly probable that substrates of MTK1-bound ERK1/2 will be unique to the role of HRG in T-47D breast cancer cells. Since ERK1/2 activity has been strongly implicated in cell proliferation and breast cancer (Clark et al., 1996; Gee et al., 2001; Xing and Imagawa, 1999; Yang et al., 2008), our results suggest that MTK1 may be required for signaling events that promote heregulin-dependent breast cancer cell proliferation.

The organization of the proteins associated with MTK1 is schematically represented in figure 17. A question that remains to be addressed is whether these proteins associate directly with MTK1. The MTK1 and GIT1 association appears to be indirect, since MTK1 was not detected by immunoblot analysis from GIT1 immunoprecipitation experiments (Figure 16C & D). Similarly, MTK1 and Grb2 association appears to be
indirect, since MTK1 was not detected by immunoblot analysis from Grb2-GST fusion protein pull-downs (Figure 16B). Although direct association between MTK1 with GIT1 and Grb2 was not observed, direct association of MTK1 with activated ERK1/2 and tyrosine phosphorylated Shc is possible. Inspection of the MTK1 amino acid sequence reveals multiple putative D domains, but no DEF motifs, that might mediate the interaction between ERK1/2 and MTK1. The D domain is a MAP kinase docking site consisting of the amino acid motif, LXL with a cluster of basic amino acids (Bardwell and Thorner, 1996; Kallunki et al., 1994; Sharrocks et al., 2000; Tanoue et al., 2000; Yasuda et al., 1999), while the DEF motif is another docking motif for MAP kinases that consists of the amino acids FXFP (Fantz et al., 2001; Galanis et al., 2001; Jacobs et al., 1999; MacKenzie et al., 2000; Murphy et al., 2002).
FIGURE 17. A SCHEMATIC REPRESENTATION OF THE ORGANIZATION OF PROTEINS ASSOCIATING WITH MTK1.
While the interaction between Shc and Grb2 has been well characterized, we demonstrate for the first time that the amino-terminal SH3 domain of Grb2 mediates the interaction with GIT1. Although we have not explored the domain within GIT1 that interacts with Grb2, it is likely a proline-rich region and not a tyrosine phosphorylated region, since the SH2 domain of Grb2 does not interact with GIT1. The interaction between ERK1/2 and GIT1 as demonstrated by co-immunoprecipitation could also be mediated by D domains on GIT1. How the organization of Grb2, Shc, GIT1 and ERK1/2 accommodates the recruitment of MEK is not known. The ability of PD98059 to inhibit ERK1/2 activity that is bound to MTK1 demonstrates that MEK participates in this signaling complex, albeit transiently. In conclusion, much is known about the Ras-Raf-MEK-ERK1/2 signaling pathway. The functional significance of the MTK1-GIT1-ERK1/2 signaling complex will be revealed once there is identification of MTK1-bound ERK substrates. However, sustained phosphorylation of the ERK motif within those substrates will likely be a characteristic that differentiates MTK1-GIT1-ERK1/2 from Ras-Raf-MEK-ERK1/2 substrates.
CHAPTER 4

OVERALL CONCLUSIONS AND FUTURE DIRECTION

4.1 OVER ALL CONCLUSIONS

The data presented in this dissertation define a novel interaction between endogenous MTK1, HER2/HER3, ERK1/2, Shc, Grb2, p85, f-actin and GIT1 in response to HRG stimulation in human epithelial breast cancer cells. These HRG dependent protein interactions led to the hypothesis that MTK1 is regulated by RTK signaling. MTK1 was immunoprecipitated and the HRG dependent MTK1, pY1289 HER3, activated ERK1/2, tyrosine phosphorylated GIT1, tyrosine phosphorylated Shc 52 kDa, Grb2 and actin associations were identified through immunoblot analysis. Additionally, the MTK1, HER3 and GIT1 associations were confirmed through LC-MS/MS experiments with a 99.9% confidence for positive identification. The association between MTK1 and HER3 was only observed with HRG stimulation. This observation was seen in both immunoblot analysis and LC-MS/MS experiments. Furthermore, the association of MTK1 and p85 of PI3K was only observed with HRG stimulation. Moreover, a constitutive association between MTK1, ERK1/2, GIT1, Shc, Grb2 and actin was also observed. Additionally, Grb2 fusion protein pull-down experiments revealed GIT1 to associate strongly with the amino-terminal SH3 domain of Grb2, while GIT1 was also observed to associate with full length Grb2.
Further studies revealed that HER2 was required for the MTK1/HER3 association. Pretreatment of the MCF-7 cells with 50 nM lapatinib inhibited the association between MTK1 and tyrosine phosphorylated HER3. HER2 did not immunoprecipitate with MTK1 in response to HRG stimulation. This suggested that the tyrosine phosphorylation of HER3, that was associated with MTK1, was performed transiently by HER2. Furthermore, HRG stimulation of HER2 negative but HER3 positive MDA-MB-231 cells failed to produce an MTK1 and HER3 association. These results further suggested HER2 was required for the HRG dependent MTK1/HER3 association.

Additional studies revealed that f-actin was required the MTK1/HER3 association. Pretreatment with cytochalasin D (an inhibitor of actin polymerization) significantly inhibited the HRG dependent MTK1/HER3 association. Actin polymerization is required for cell migration and additional studies revealed that HRG stimulation led to the extracellular acidification and migration of MCF-7 cells. MTK1 was identified as being required for both of these HRG dependent functional readouts. Transfection of MCF-7 cells with MTK1 specific siRNA significantly inhibited the extracellular acidification and migration in response to HRG stimulation in MCF-7 cells.

Breast cancer is the most commonly diagnosed form of cancer for women in the United States (CDC 2009 Figure 2). As previously mentioned, the risk factors associated with
breast cancer include but are not limited to age, gender, diet, sedentary life style, extensive use of hormonal replacement therapy, chemical environmental exposures and genetic predisposition (Mitra et al., 2004; Thomson, 2012; Welnicka-Jaskiewicz and Jassem, 2003). Invasive breast cancers are associated with over 90% of breast cancer related mortality (Wang, 2010). Additionally, mortality is not generally associated with the primary tumor, but instead is a result of metastasis to other organs (Scully et al., 2012). Early diagnosis and development of new pharmaceuticals to inhibit or prevent metastasis is paramount for the prevention of breast cancer related mortality.

HER2 has been a molecular target in tamoxifin resistant breast cancer for several years. The efficacy of trastuzumab (Herceptin) and lapatinib (Tykerb) in fighting HER2 resistance tumors has been good, however resistance still persists (Bedard et al., 2009; Engel and Kaklamani, 2007; Esteva and Pusztai, 2005; Scaltriti et al., 2009; Stern, 2012). HER3 protein expression was shown to be up-regulated with lapatinib treatment, compensating for HER2 inhibition, and HER3 phosphorylation occurred by residual HER2 expression limiting the efficacy of lapatinib treatment (Garrett et al., 2011). Therefore, HER3 over-expression and recovery of phosphorylation appears to be a compensatory mechanism in response to drug targeting of HER2.

Additionally, Vaught et al. showed that HER3 is required for HER2 induced pre-neoplastic changes and tumor formation in breast epithelium. (Vaught et al., 2012).
These observations have shed new light on HER3 as an attractive new molecular target for treating HER2 positive breast cancer.

Metastasis, not the primary tumor, is the major lethal component in most cancers. Therefore, it is paramount that we identify proteins that promote this deadly hallmark of cancer. By identifying these proteins we can develop new pharmaceuticals that disrupt protein/protein interactions required for metastasis. The MTK1/HER3 heterodimer is a new and previously uncharacterized protein complex in the HRG dependent HER2/HER3 signaling pathway. In addition to targeting HER3, MTK1 is a promising new molecular target for the treatment of HER2/HER3 positive breast cancers. The development of pharmaceuticals that target MTK1 and block the association with HER3, f-actin, GIT1, ERK1/2 or Shc may be effective in blocking metastasis in HER2/HER3 positive breast cancers and therefore improve the patient’s prognosis and chances of survival.
4.2 FUTURE DIRECTION

Migrating cells require coordinated actin cytoskeletal reorganization that includes actin polymerization and de-polymerization in addition to association with actin binding proteins (Cooper, 1991; Doherty and McMahon, 2008). Some of the structures formed from the actin cytoskeleton in migrating cells are filopodia, lamellipodia and lamella. Filopodia are long slender cytoplasmic extensions that protrude past the leading edge of a migrating cell (Figure 18). Filopodia consist of actin filaments cross-linked to actin binding proteins and form from the lamellipodia. Lamellipodia are thin foot like cytoskeletal actin structures located on the leading edge of a migrating cell (Figure 18). Furthermore, the lamella also consists of actin filaments and is located adjacent to the leading edge of a migrating cell (Figure 18). Both the lamellipodia and lamella are required for cell migration (Burnette et al., 2011). However, how lamellipodia and lamella interact mechanistically is still not fully understood.

MTK1 localizes apically toward the leading edge of the lamella of migrating MCF-7 breast cancer cells. Preliminary data acquired from confocal z-stacked immunofluorescence images using MTK1 specific antibodies revealed MTK1 to localize apically within the region of the lamella (Figure 19). Z-stacked confocal images were obtained from migrating MCF-7 breast cancer cells. The z-plane images were acquired beginning apically and finishing toward the basal lateral side of the cell. A total of seven
z-plane images were taken at 0.5 µm intervals. Apical images 1-4 reveal MTK1 to localize and concentrate apically in the region of the lamella (Figure 19). The basal lateral images 5-7 revealed MTK1 to be expressed ubiquitously in the cytoplasm (Figure 19). However, MTK1 was not concentrated in the basal lateral region like observed in the apical region of the cell (Figure 19). Image 8 is a cartoon tracing of image 7 showing the location of the nucleus, lamella and lamellipodia in relation to the entire cell.

Does MTK1 localize with the lamella at different stages of cell migration? Lamella formation is required for cell migration (Burnette et al., 2011). If MTK1 is required for lamella formation, this would explain why siRNA specific knock down of MTK1 inhibits HRG induced cell migration in MCF-7 cells (Sollome et al., 2013). Future experiments utilizing time lapsed imaging will need to be performed to map the localization of MTK1 at different stages of cell adhesion and detachment during cell migration. This will give us a better understanding of how MTK1 interacts within the lamella region and regulates HRG induced cell migration in breast cancer.
Migrating cells require coordinated actin cytoskeleton reorganization that includes actin polymerization and de-polymerization in addition to association with actin binding proteins. Filopodia are slender long cytoplasmic extensions that protrude past the leading edge of the lamellipodia in migrating cells. Filopodia consist of actin filaments cross-linked to actin binding proteins. The lamella also consists of actin filaments and is located adjacent to the leading edge of a migrating cell. Lamella formation is required for cell migration. Preliminary data showed MTK1 to concentrate and localize apically in the region of the lamella in migrating MCF-7 breast cancer cells.
FIGURE 19. MTK1 LOCALIZES APICALLY AT THE LAMELLA TOWARD THE LEADING EDGE OF MIGRATING MCF-7 BREAST CANCER CELLS.

A total of seven z-stack images were taken at 0.5 µm intervals. The confocal immunofluorescence z-stacked images begin from the apical (image 1) through to the basal lateral side (image 7) of the cell. MTK1 is expressed ubiquitously throughout the cytoplasm. Images 1-4 reveal MTK1 to be localized and concentrated apically at the lamella of the cell. Image 8 is a cartoon tracing of image 7 showing the location of the nucleus, lamella and lamellipodia in relation to the entire cell.
Actin polymerization and subsequent cell migration is also dependent on microtubule formation (Doherty and McMahon, 2008). Microtubules are formed through polymerization of tubulin proteins (Weisenberg et al., 1976). The tubulin superfamily consists of five family members, alpha, beta, gamma, delta and epsilon, the most common of these is the alpha-tubulin and beta-tubulin proteins which together form microtubules (Oakley, 2000). Preliminary experiments show MTK1 to co-localize with beta-tubulin apically at the region of the lamella and perinuclearly in a bloodshot eye pattern (Figure 20). MCF-7 cells were fixed and co-stained with MTK1 (red) and beta-tubulin (green) specific antibodies. Z-stacked images were taken at 0.5 µm intervals. MTK1 (red) immunofluorescence z-stacked images were acquired beginning apically, image 1, and ending basal laterally, image 3, (Figure 20). Image 1 shows MTK1 to localize perinuclearly in a bloodshot pattern. Images 2 and 3 show MTK1 to concentrate and localize apically in the region on the lamella (Figure 20). The z-stacked images 4-6 are the same as images 1-3, only they display both the MTK1 (red) channel and the beta-tubulin (green) channel together. Co-localization of MTK1 and beta tubulin was observed by merge of the two colors to form yellow. Images 4-6 reveal MTK1 and beta tubulin to co-localize (yellow color) in the area of the lamella and perinuclearly in a bloodshot pattern (Figure 20). Beta tubulin was also identified as immunoprecipitating with MTK1 from MCF-7 cells stimulated with HRG. LC-MS/MS was performed to confirm the association of beta tubulin with MTK1, with 23% total protein coverage identified for tubulin. The following peptides were sequenced multiple times and
identified with 99.9% confidence for positive identification of beta tubulin (EVDEQMLNVQNK and GHYTEGAELVDSVLDVVR).

Future experiments utilizing time lapsed imaging will need to be performed to further characterize the interaction of MTK1 and beta tubulin in the area of the lamella and perinuclearly at different stages of cell adhesion and detachment during cell migration. As with lamella characterization, these experiments will give us a better understanding of how MTK1 interacts beta tubulin and regulates HRG induced cell migration in breast cancer.
FIGURE 20. MTK1 LOCALIZES WITH BETA-TUBULIN PERINUCLEARLY AND APICALLY NEAR THE LAMELLA IN MCF-7 CELLS.

MCF-7 cells were fixed and co-stained with MTK1 (red) and beta-tubulin (green) specific antibodies. Preliminary experiments show MTK1 to co-localize with beta-tubulin apically at the area of the lamella and perinuclearly in a bloodshot eye pattern. Image 1 shows MTK1 to localize perinuclearly in a bloodshot eye pattern. Images 2 and 3 show MTK1 to localize in the region of the lamella. Image 4 shows MTK1 and beta
tubulin (yellow) to co-localize perinuclearly in a bloodshot eye pattern. Images 5 and 6 show MTK1 and beta tubulin (yellow) to co-localize in the region of the lamella.

In addition to localizing perinuclearly and in the region of the lamella, MTK1 also appears to localize in a punctate pattern within the nucleus. Immunofluorescence experiments using MCF-7 cells reveal, in image 1 (red), MTK1 to localize in the nucleoli of the nucleus (Figure 21). Nucleoli are localized within the nucleus and unlike cytoplasmic organelle, they are not held together by membranes. In fact, nucleoli are best described as multi-molecular complexes held together by their component proteins and nucleic acids (Shaw and Brown, 2012). One of the component proteins that is highly expressed in the nucleoli and involved in ribosome biogenesis is nucleolin (Shaw and Brown, 2012). The punctate nuclear structures that stained positive for MTK1 appeared to be nucleoli. To determine whether these structures were nucleoli, MCF-7 cells were co-stained for MTK1 (red) and nucleolin (green) (Figure 21). Image 2 (green) revealed that the punctate structures were in fact nucleoli (Figure 21). Image 3 was acquired using frozen HER2/HER3 positive human breast cancer tissue and showed MTK1 (red) to localize in a punctate pattern in the nucleus which appears to be nucleoli (Figure 21). Taken together, these preliminary data show that MTK1 localizes within the nucleoli of the nucleus in both human MCF-7 breast cancer cells and human HER2/HER3 positive breast cancer tissue.
Future experiments will need to be performed in breast cancer cells and human tissue to characterize the function of MTK1 within the nucleoli structures. Proteins within the nucleoli initiate ribosomal biogenesis (Shaw and Brown, 2012), so it is tempting to postulate that MTK1 has a role in this process.

Figure 21
FIGURE 21. MTK1 LOCALIZES IN THE NUCLEOLI OF THE NUCLEUS.

IMAGE 1 (RED) SHOWS MTK1 TO LOCALIZE IN PUNCTATE STRUCTURES OF THE NUCLEUS.

Image 2 is specific for nucleolin (green) and reveals MTK1 localizes within the nucleoli of the nucleus. Image 3 reveals MTK1 (red) to localize in a punctate pattern in the nucleus of HER2/HER3 positive breast cancer tissue. This punctate staining appears to be within nucleoli in the breast cancer tissue.
Other future experiments include identifying uncharacterized substrates of MTK1. The data presented here in this dissertation reveal scaffolding properties of MTK1 with HER3, ERK1/2, GIT1, p85 of PI3K, Shc, Grb2 and f-actin in response to HRG stimulation in human breast cancer cells. Is MTK1 kinase active in response to HRG stimulation? Any of these proteins that interact with MTK1 could be potential substrates of MTK1. We currently do not have a kinase assay to measure MTK1 kinase activity. Therefore, to answer this question a kinase assay must be developed that allows us to characterize downstream substrates of MTK1.

To address this issue, an ATP binding pocket mutant for MEKK4, the mouse homolog of MTK1, has been created. Methionine 1408 in the ATP binding pocket has been mutated to glycine (M1408G). This creates a larger binding pocket that in theory is only specific for an ATP analog. The ATP analog terminal γ phosphate is replaced with a terminal γ sulphate group. In a kinase reaction, the ATP analog terminal sulphate would be transferred to the substrate; the substrate could then be treated with the chemical p-nitrobenzyl mesylate (PNBM). This creates a thiophosphate-ester motif that can be recognized with a thiophosphate-ester specific rabbit antibody. These experiments will determine whether HRG stimulation initiates MTK1 kinase activity. They will also identify new uncharacterized substrates of MTK1.
The severe combined immunodeficient (SCID) mice are the perfect candidate for quantifying tumor growth potential of human cancer cells. SCID mice are deficient in functional T and B lymphocytes and subcutaneous transplantation of MCF-7 cells have been shown to form tumors in these mice (Zhai et al., 1992). Knockdown of MTK1 inhibited HRG dependent extracellular acidification and cell migration in MCF-7 cells (Sollome et al., 2013). To further characterize MTK1 in tumor formation, future experiments injecting SCID mice with MTK1 knockdown MCF-7 cells need to be performed. Tumor formation and size can be assessed in response to MTK1 knockdown injected mice. Additionally, peripheral tumor pH can be quantified in these mice to determine effects of MTK1 knockdown on tumor peripheral acidification as observed in HRG dependent MCF-7 cell cultured media.

Pretreatment of MCF-7 cells with cytochalasin D inhibited the association of MTK1 and HER3 in response to HRG stimulation (Sollome et al., 2013). Cytochalasin D inhibits actin polymerization, suggesting f-actin is required for MTK1 and HER3 to associate. Additionally, deletion of the MEKK4 actin interacting region (AIR) inhibited the association of MEKK4 and actin. This suggests a direct association between MTK1 and actin. Therefore small molecule inhibitors that target the MTK1 AIR could be developed and tested to inhibit MTK1/actin association and subsequent MTK1/HER3 association. Development of inhibitors that block MTK1/HER3 association may be effective additions to lapatinib and trastuzumab in the treatment of HER2/HER3 positive breast cancer therapy.
HRG stimulation led to the extracellular acidification and cell migration in both MCF-7 and T-47D cells. Dysregulated pH is an emerging hallmark of cancer; in fact cancer cells have a reversed pH gradient with an increased intracellular pH that is higher than the extracellular pH (Webb et al., 2011). As mentioned previously, cancer cells have increased glycolytic metabolism leading to acid loading and as a result, excess protons are excreted from the cell by the up-regulation of proton transporters (Stock et al., 2005). This reversed pH gradient potentiates cancer progression through increased proliferation, evasion of apoptosis, metabolic adaptation, migration and invasion (Webb et al., 2011).

In MCF-7 cells, knockdown of MTK1 using siRNA inhibited both the extracellular acidification and cell migration. (Sollome et al., 2013). There are many different proton transporters that could be responsible for the HRG induced extracellular acidification. These transporters need to translocate to the plasma membrane to facilitate proton export. Cancer cells have increased expression levels of H⁺-ATPase’s, the Na⁺-H⁺ exchanger (NHE1) and monocarboxylate-H⁺ efflux cotransporter (MCT) (Webb et al., 2011). The increased excretion of protons leads to extracellular acidification which can activate proteases that promote cell migration and invasion (Webb et al., 2011). Both H⁺-ATPase’s and NHE1 transporters are expressed in MCF-7 cells and likely contribute to the extracellular acidification in response to HRG stimulation. However, preliminary data suggests that MCT proteins are a likely candidate that should be investigated first. The MCT proteins have a high affinity for lactate, which is increased in cancer cells due
to the cancer cell’s ability to convert pyruvate to lactate (Webb et al., 2011). Protons are co-transported out of the cell with lactate contributing to the extracellular acidification.

Media taken from MCF-7 cells stimulated with HRG for 48 hours were analyzed for both pH changes and lactate excretion. As expected, HRG induced extracellular acidification as observed in previous experiments. Additionally, lactate excretion was shown to be significantly elevated in HRG stimulation (Figure 22). Since protons are co-transported with lactate, MCT proteins may contribute the HRG dependent extracellular acidification in MCF-7 cells. The quantification of the lactate excretion was performed by Fei Zhao in the lab of Dr. Walter Klimecki at the University of Arizona. Future lactate assay experiments need to be repeated to quantify lactate excretion and validate these results. Additionally, the MCT proteins are also known to be expressed at the leading edge of migrating cells (Webb et al., 2011). Localization of MCT proteins should be characterized to further validate the role of these transporters in extracellular acidification. This can be accomplished by immunofluorescence experiments. It would also be interesting to knockdown MTK1 and measure lactate excretion in response to HRG. The prediction would be that knocking down MTK1 would decrease lactate excretion.

Hyaluronic acid (HA) excretion is another possible mechanism that could contribute to the HRG dependent extracellular acidification. HA is enriched in many types of cancer
and is linked to the NHE1 transporter and subsequent proton transport (Bourguignon, 2008). To determine if HA was involved, T-47D breast cancer cells were stimulated with HRG for 48 hours. Media was then collected and used for an ELISA assay to quantify HA excretion. The preliminary data revealed that HRG stimulation significantly increased HA excretion in T-47D cells (Figure 23). Additionally, pre-treatment with 250 nM lapatinib significantly inhibited HA excretion (Figure 23). These data suggest HA is increased in response to HRG stimulation and may contribute to extracellular acidification. Additional experiments need to be repeated in the future to further validate the role of HA excretion in HRG induced extracellular acidification. Additionally, it would be interesting to knockdown MTK1 and measure HA excretion in response to HRG. Like lactate, the prediction would be that knocking down MTK1 would decrease HA excretion.
FIGURE 22. LACTATE EXCRETION IS SIGNIFICANTLY ELEVATED WITH HRG STIMULATION IN MCF-7 CELLS.

In addition to extracellular acidification, HRG stimulation significantly increased levels of lactate excretion in MCF-7 cells. The concentration of lactate in cell media was reported in mM of lactate per 10⁶ cells. Media lactate levels were 354.3 for control, 554.2 for HRG and 403.4 for EGF.
FIGURE 23. HYALURONIC ACID EXCRETION IS SIGNIFICANTLY ELEVATED WITH HRG STIMULATION IN T-47D CELLS.

An ELISA was used to quantify HA excreted into media of T-47D cells. HRG stimulation significantly increased levels of HA excretion. Pretreatment with 250 nM lapatinib significantly inhibited HA excretion.
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