NOTE TO USERS

ROLE OF ERBB3 DURING MAMMARY TUMORIGENESIS

By

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ABSTRACT

Overexpression of the Neu/ErbB-2 receptor tyrosine kinase is implicated in the genesis of human breast cancer. Previous studies have observed elevated levels of endogenous ErbB3 protein in Neu-induced tumors. Although it has been suggested that the aberrant co-expression of Neu and ErbB3 may play a critical role in the induction of human breast tumors, the biological significance and the molecular mechanism of ErbB3 regulation during Neu–mediated tumorigenesis remains unclear.

The results of this thesis demonstrate that the ability of ErbB3 to be constitutively phosphorylated and to regulate cell cycle progression through the activation of the PI-3K/mTOR pathway fully depends on the activity of Neu. Both PI-3K and mTOR in turn are involved in sustaining high levels of ErbB3 protein by increasing its stability. A mutant of ErbB3 unable to recruit and activate PI-3K (ErbB3-6F), blocks Neu-induced transformation, disrupts the mTOR/4EBP1 pathway and leads to apoptotic cell death. This strongly suggests that ErbB3's role in these tumors is to provide cell survival signals by recruiting the p85 regulatory unit of PI-3K and coupling overexpressed Neu to the PI-3K/mTOR/4EBP1 pathway.

This thesis further demonstrates that the mTOR inhibitor rapamycin, delays the onset and inhibits the growth of Neu-induced mammary tumors in transgenic mice. The rapamycin-induced tumor inhibition correlates with downregulation of ErbB3, S6K, 4EBP1 and cyclin D1. I also show that the ErbB3-6F mutant exhibits antitumor activity *in vivo* by preventing tumor growth and facilitating tumor regression. Therefore, the antitumor activity of the rapamycin analogue, CCI-779 in human breast cancer may be mediated, in part, through the inactivation of ErbB3 thereby inhibiting the critical cell survival signals necessary for transformation. Taken together these results strongly suggest that Neu requires ErbB3 to drive mammary transformation and that the ErbB2/ErbB3 heterodimer may function as an oncogenic unit to recruit distinct yet complimentary signaling pathways that cooperate during mammary tumor progression. The results of this thesis provide a molecular basis for the selective targeting of the ErbB3/PI-3K/mTOR signaling pathway in the treatment of HER2-mediated human breast malignancies in which HER3 is overexpressed.

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CONTRIBUTIONS BY OTHERS

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LIST OF ABBREVIATIONS

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
MAPK	Mitogen Activating Protein Kinase
MMLV	Moloney murine leukemia virus
MMTV-LTR	Mouse mammary tumor virus-long terminal repeat
mT	Middle T antigen
mTOR	Mammalian target of rapamycin
NDL	Neu Deletion
NeuKD	Neu Kinase Dead
PGK	Phosphoglycerate kinase
PI-3K	Phosphatidylinositol 3-kinase
RTK	Receptor tyrosine kinase
S6K	p70 S6 kinase
4EBP1	4E binding protein 1

CHAPTER 1

Introduction

1.1 MAMMARY TUMORIGENESIS

Mammary tumor development is a multi-step process which begins with a benign hyperplasia and ends with an invasive tumor capable of metastasizing to distant organs. During this process, the cells escape normal regulatory processes which are necessary for the full malignant phenotype. Cancer cells must acquire proliferative potential and divide continuously. To accomplish this, cells must overcome contact inhibition and cellular checkpoints, which normally would induce apoptosis. The primary tumor also restructures its supply of nutrients and oxygen through the formation of new vessels. Finally, the tumor cells gain several new features including the ability to migrate and invade distant tissues.

The most frequently observed genetic abnormality associated with human breast cancer involves DNA amplification and/or overexpression of genes responsible for the generation of proliferative signals. The products of these genes include growth factors, growth factor receptors, cell cycle regulatory proteins and nuclear transcription factors. Epidermal growth factor (EGF) receptor tyrosine kinases (RTKs) are critical in the genesis of human breast cancer and are the focus of this thesis.

1.2 ROLE OF EGFR FAMILY IN MAMMARY TUMORIGENESIS

The epidermal growth factor receptor (EGFR) family of RTKs includes four members, EGFR/ErbB1/HER1 (Ullrich et al., 1984), ErbB2/Neu/HER2 (Bargmann et al., 1986b; Yamamoto et al., 1986) ErbB3/HER3 (Kraus et al., 1993; Plowman et al., 1990), and ErbB4/HER4 (Plowman et al., 1993a). The main function of these receptors is to send extracellular signals to the cell. All ErbBs have in common a highly glycosylated extracellular ligand-binding domain with two cystein-rich clusters, a single transmembrane region and a cytoplasmic protein tyrosine kinase domain. Activation of

these receptors is regulated by expression of their respective ligands which are members of the EGF-related peptide growth factor family.

Ligand binding to ErbB receptors induces formation of homo-and heterodimers. This leads to activation of the intrinsic kinase activity of the component monomers and subsequent phosphorylation of specific tyrosine residues with the cytoplasmic tail. These phosphorylation residues serve as docking sites for Src homology 2 (SH2) and phophotyrosine-binding (PTB)-domain-containing cytoplasmic signaling molecules, whose recruitment leads to activation of appropriate downstream signaling cascades, including mitogen activated kinase (MAPK) and phosphatidylinositol-3 kinase (PI-3K) (Figure 1.1).

Several clinical studies have implicated the ErbB-driven autocrine growth pathway in the development and progression of human breast cancer; its blockade therefore is a promising therapeutic strategy, and several candidate drugs are currently undergoing clinical trials.

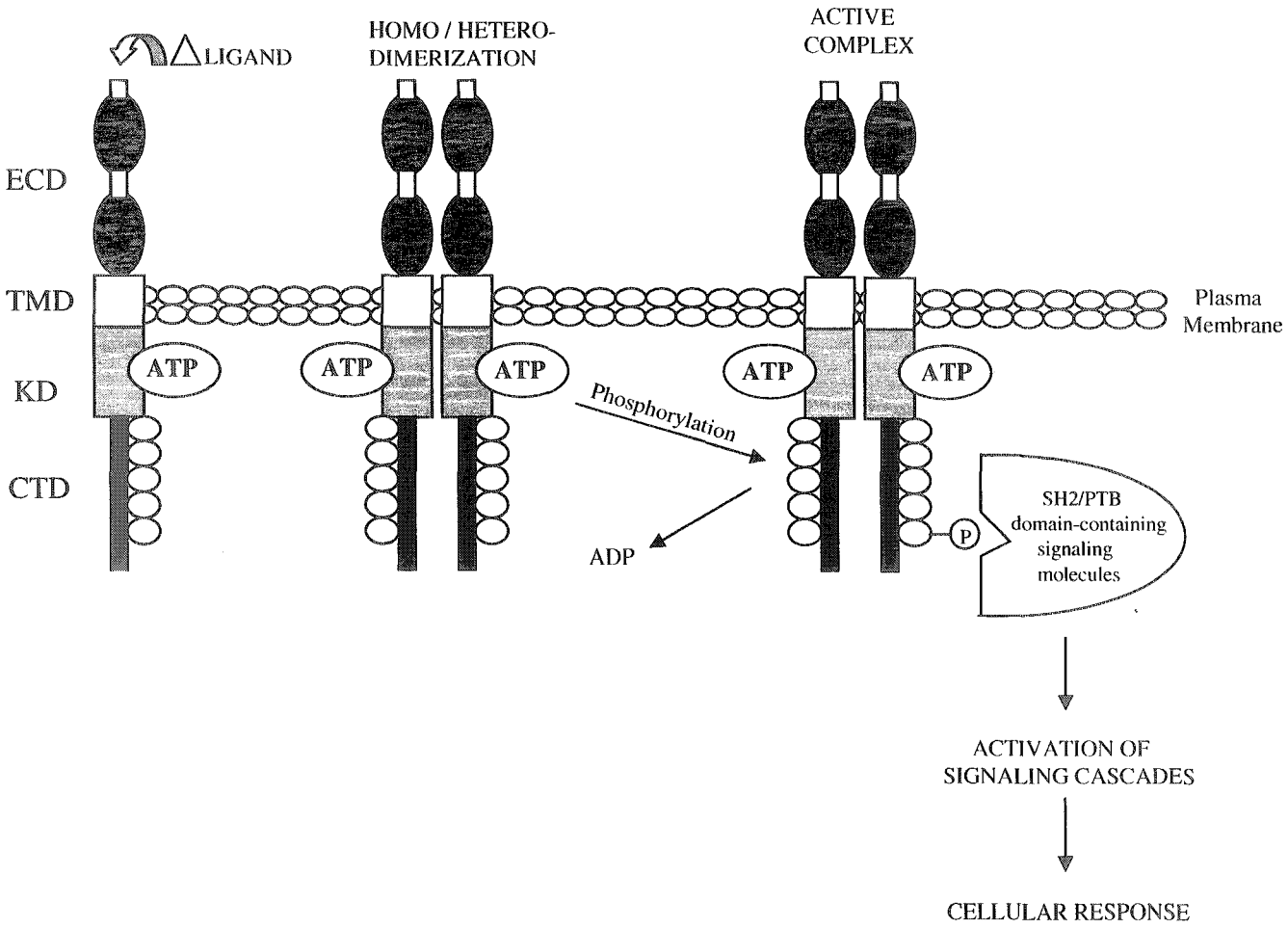
Early investigations by Sainsbury et al revealed a strong correlation between EGFR overexpression and poor clinical outcome for the patient (Sainsbury et al., 1987). Similarly, Klijn et al suggested that EGFR overexpression was strongly associated with a higher risk of cancer relapse and death (Klijn et al., 1992). This finding however, was only weakly supported in longer follow-up studies (Bieche et al., 2003; Dittadi et al., 1993; Robertson et al., 1996).

Like EGFR, there is very little known about the correlation between ErbB3 and the genesis of human breast cancers. Studies have shown that ErbB3 overexpression has been observed in 13-29% of human breast cancers (Gasparini et al., 1994; Lemoine et al., 1992; Quinn et al., 1994) and this elevation is not due to gene amplification (Kraus et al., 1989; Lemoine et al., 1992). Very few studies have studied correlation between ErbB3 overexpression and patient prognosis. Bieche et al however, have shown that patients overexpressing ErbB3 experienced a shorter relapse-free survival (RFS) relative to patients with normal expression of the gene. Interestingly, RFS was not significantly influenced by the status of EGFR and ErbB2 expression (Bieche et al., 2003).

There is an extensive literature on the role of ErbB2 in breast cancer. ErbB2 amplification and overexpression has been found in 15-25% of breast cancers, and its

Figure 1.1. Activation of Epidermal Growth Factor Receptor Tyrosine Kinases. The epidermal growth factor receptor (EGFR) family of RTKs includes four members, EGFR/ErbB1/HER1, ErbB2/Neu/HER2, ErbB3/HER3 and ErbB4/HER4. The main function of these receptors is to send extracellular signals to the cell. All ErbBs have in common a highly glycosylated extracellular ligand-binding domain (ECD) with two cystein-rich clusters, a single transmembrane domain (TMD), a kinase domain (KD) and a cytoplasmic protein tyrosine kinase domain (CTD). Ligand binding to ErbB receptors induces formation of homo-and heterodimers. This leads to activation of the intrinsic kinase activity of the component monomers and subsequent phosphorylation of specific tyrosine residues within the cytoplasmic tail. These phosphorylation residues serve as docking sites for Src homology 2 (SH2) and phophotyrosine-binding (PTB)-domain-containing cytoplasmic signaling molecules, whose recruitment leads to activation of specific downstream signaling cascades leading to an appropriate cellular response.

Figure 1.1


LIGAND
HOMO / HETERO-
DIMERIZATION
ACTIVE
COMPLEX
ECD
TMD
KD
CTD
Plasma
Membrane
ATP
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SH2/PTB
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ACTIVATION OF
SIGNALING CASCADES
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presence is strongly associated with poor prognosis (Press et al., 1993; Slamon et al., 1987; Slamon et al., 1989). ErbB2 overexpression predicts poor response to both chemo- (Thor, 2001; Thor et al., 1998) and hormonal therapy (Stal et al., 2000). For this reason several anti-ErbB2 cancer therapies have been developed and tested (summarized in (Atalay et al., 2003)). Trastuzuman (Herceptin), a humanized anti-ErbB2 monoclonal antibody has been approved in combination with paclitaxel for the treatment of patients with ErbB2-overexpressing metastatic breast cancer. The tyrosine kinase inhibitor TAK-165 is currently being evaluated in a phase I trial in HER-2 metastatic breast cancer (Naito et al., 2002; Yoshida et al., 2002). Several ongoing vaccination strategies aim to improve specific immune responses to tumor-specific antigen. A phase I/II clinical trial is currently assessing the tolerability of AutoVac HER-2 DNA pharmaccine (AutovacTM Pipeline [on-line], 2004). In addition a phase I gene therapy clinical trial using 5E1A (adenovirus type 5 which represses the HER-2 gene), has been studied in advanced stage breast cancer (Wang and Hung, 2001).

Given its increasing clinical importance, a better understanding of the molecular basis of EGFR family mediated tumorigenesis will identify important targets for therapy, as well as identify those patients who benefit most from certain interventional strategies.

1.3 THE NEU ONCOGENE

The neu oncogene was first identified as an activated oncogene. Purified DNA from tumors of carcinogen-treated rats was able to induce malignant growth of NIH 3T3 cells after transfection (Shih et al., 1979; Shih et al., 1981) suggesting that the DNA contained gene(s) with oncogenic potential. Secondary neuroblastoma transfectants were injected into mice in order to identify the protein responsible for the transformation. The crude antisera from tumor bearing mice precipitated a phosphoprotein of 185 kDa (Padhy et al., 1982). This transforming protein was then shown to be similar to growth factor receptors and was named 'neu' for the neuroglioblastoma from which it was derived (Schechter et al., 1984). Southern blot analysis indicated that the genomic DNA shared a high degree of similarity with the v-erbB oncogene and that the 185 kDa tumor antigen shared immunologic determinants with EGFR.

The neu cDNA was subsequently cloned and comparison of the transforming and wild type forms of neu revealed a single amino acid substitution in the transmembrane domain of ongonenic neu, converting a valine residue to glutamic acid (Bargmann et al., 1986). This oncogenic neu was referred as NeuNT. The consequences of this mutation for Neu activation is discussed in subsequent sections.

1.4 EGFR FAMILY OF LIGANDS

Under normal physiological conditions, activation of the ErbB receptors is controlled by their respective ligands which are produced as transmembrane precursors where proteolysis leads to production of soluble growth factors. These ligands are members of the EGF-related peptide growth factor family and can be subdivided into three groups based on their abilities to modulate the catalytic activity of the four EGFR family members. The first group includes EGF, amphiregulin and transforming growth factor-α, which bind specifically to EGFR (Normanno et al., 1994). The second group betacellulin, heparine-binding EGF and epiregulin possess dual specificity and therefore bind EGFR and ErbB4 (Riese and Stern, 1998). The third group consists of neuregulins (NRG), also known as Neu differentiation factors (NDFs) (Peles et al., 1992; Wen et al., 1992), and forms two subgroups based on their capacity to bind ErbB3 and ErbB4 (NRG-1 and NRG-2) or only ErbB4 (NRG-3 and NRG-4) (Carraway, III et al., 1994; Plowman et al., 1993b; Sliwkowski et al., 1994).

Despite the abundance of receptors identified for EGFR, ErbB3 and ErbB4, a fully characterized ligand that binds directly to Neu has yet to be discovered. However, there are candidates that do exist such as Neu-activating factor (NAF) (Dobashi et al., 1991; Samanta et al., 1994) which has been shown to interact with Neu expressed in insect cells and to stimulate dimerization and tyrosine phosphorylation of Neu expressed in mammalian cells (Samanta et al., 1994). Another transmembrane molecule referred to as ascites sialoglycoprotein-2 (ASGP-2) (Carraway et al., 1993) has been proposed as a native ligand for ErbB2 (Carraway et al., 1997).

1.5 ERBB-2/NEU DIMERIZATION AND ACTIVATION

Studies have shown that dimerization of two Neu receptor monomers is one of the first events to occur during receptor activation (Heldin, 1995; Schlessinger and Ullrich, 1992). The receptor has been shown to be activated by ligand binding, by overexpression and mutation leading to constitutive activation.

The principle mechanism by which ErbB2 is activated in human breast cancer is through amplification and overexpression. Several studies have shown that expression of high levels of wild-type ErbB2 and Neu in rat fibroblast cells induces transformation (di Fiore et al., 1987; Di Marco et al., 1990; Hudziak et al., 1987). Bargmann and Weinberg have suggested the importance of overexpression by demonstrating that expression of Neu at low levels does not lead to transformation of cultured mouse fibroblasts (Bargmann et al., 1986a). Indeed studies have shown that dimerization of Neu increases with increasing expression of Neu by resulting in a greater frequency of inter-receptor collision (Cao et al., 1992; Samanta et al., 1994).

Early studies identified that the effects of the point mutation seen in rat neu were mediated by dimerization. Weiner et al used nondenaturing gel electrophoresis with cell lysates prepared form cells overexpressing wild type Neu or NeuNT forms of p185 and found that the majority of p185 existed in a dimeric form, while wild type p185 existed in monomeric forms (Weiner et al., 1989). Further supporting the role of oligomerization of neu oncogene, Lofts et al co-transfected short neu transmembrane sequences lacking both the intracellular and extracellular domains (Lofts et al., 1993). These transfections inhibited oligomerization and the transforming potential of full-length p185neu oncogene. This strongly suggested that the formation of arranged oligomers are required for the activation of neu.

Several studies have identified the mechanism by which the transmembrane mutation observed in NeuNT leads to transformation. Although it is well accepted that this point mutation increases oligomerization and hence the activity of Neu, the mechanism by which this activation occurs remains elusive. Three models have been proposed to explain the mechanism by which the transmembrane point mutation leads to oligomerization.

The first model proposes that the adjacent 664E residues interact, while the second model proposes that the 664E residue interacts with the backbone of the adjacent receptor (Smith et al., 1996; Sternberg and Gullick, 1989). The third model proposes that the V664E mutation leads to a global conformational change within the transmembrane domain which results in a more stable dimmer formation. Molecular modeling and other structural analysis studies of the transmembrane domains of wild type Neu and NeuNT have suggested that wild type Neu contains a kinked transmembrane which prevents aggregation of the receptor whereas NeuNT is primarily helical which facilitates receptor dimerization (Brandt-Rauf et al., 1989; Brandt-Rauf et al., 1990; Brandt-Rauf et al., 1995). However, other studies have not supported this model (Sajot and Genest, 2000; Smith et al., 1996). Gullick et al argue this theory on the grounds that nuclear magnetic resonance (NMR) spectroscopy results show that partial transmembrane domain peptides from Neu and NeuNT possess identical α-helical structures (Gullick et al., 1992). Despite the controversy as to the mechanism of NeuNT activation, it is well accepted that this point mutation leads to increased dimerization, increased receptor tyrosine kinase activity and increased transformation potential of the neu protooncogene.

In addition to the point mutant, mutations in the extracellular domain of the Neu oncogene have been identified. Initially, an engineered A653C substitution in the extracellular domain of Neu was shown to enhance dimerization, however, did not result in oncogenic activation of the receptor (Cao et al., 1992). Subsequent studies showed that transgenic mice overexpressing the Neu protooncogene in the mammary gland developed spontaneous activating mutations that remove, alter or insert cystein residues in the extracellular domain of the receptor (Siegel et al., 1994). These mutations lead to constitutive dimerization and activation through the formation of intermolecular disulfide bonds and enhance the oncogenic potential of the altered receptors (Siegel and Muller, 1996). Interestingly, a splice variant of ErbB2 has been cloned (Siegel et al., 1999). This splice variant is oncogenic and can induce ligand-independent activation of ErbB2. The above studies show that the Neu receptor can be activated by several different molecular mechanisms.

1.6 INTER-RECEPTOR INTERACTIONS AMONG THE EGFR FAMILY MEMBERS

Studies have shown that in addition to the formation of homodimers, the EGFR family of receptors also have the capacity to heterodimerize with one another (Carraway, III and Cantley, 1994; Earp et al., 1995; Riese and Stern, 1998). Receptor dimerization amongst the EGFR family members have shown to be quite complex and has been demonstrated for every ErbB receptor combination. Thus, from a family of four receptors, ten complexes are possible: four homodimers and six heterodimers. Signal originating from a given receptor complex varies depending on the combination present in the dimer, leading to a repertoire of EGFR molecules for diverse signaling.

Stern and Kamps first raised the possibility of heterodimerization given the observation that EGF stimulation of Rat-1 fibroblasts led to phosphorylation of Neu (Stern and Kamps, 1988). The fact that EGF cannot bind directly to Neu suggests that the EGF-dependent activation of ErbB2 is a result of transphosphorylation through the formation of EGFR/ErbB2 heterodimers. Direct evidence suggesting heterodimerization came from studies demonstrating the presence of physical complexes between EGFR and ErbB2 in both NIH 3T3 and human breast carcinoma cells (Goldman et al., 1990; Wada et al., 1990). Co-expression of EGFR and ErbB2 in mouse fibroblast cells leads to enhanced ability of these two receptors to induce transformation (Kokai et al., 1989). ErbB3 and ErbB4 have also been shown to interact with EGFR (Kim et al., 1994; Soltoff et al., 1994; Tzahar et al., 1996) since NRG stimulation of ErbB3 (Riese et al., 1995) and ErbB4 (Cohen et al., 1996; Riese et al., 1995) leads to phosphorylation of EGFR.

ErbB2/Neu has also been shown to heterodimerize with ErbB3 and ErbB4. Transphosphorylation of ErbB2 has been observed by both ErbB3 and ErbB4 in response to NRG stimulation. Furthermore, several independent studies have detected both ErbB2/ErbB3 and ErbB2/ErbB4 heterodimers ((Karunagaran et al., 1996; Pinkas-Kramarski et al., 1996; Riese et al., 1995; Tzahar et al., 1996). Initial studies performed by Alimandi et al demonstrated the importance of the ErbB2/ErbB3 heterodimerizations by showing that co-transfection of ErbB2/ErbB3 led to transformation of NIH 3T3 cells (Alimandi et al., 1995). This finding was further supported by studies showing that

coinfection of ErbB2/ErbB3 in NIH3T3 cells led to the formation of colonies in soft agar (Wallasch et al., 1995). Subsequently, several other independent studies highlighted the importance of transphosphorylation and heterodimerization amongst the EGFR members by using mammary carcinoma cell lines expressing all four members of the EGFR family (T47D and MCF7 cells). They used a single chain antibody that specifically retains ErbB2 in the endoplasmic reticulum. Both EGFR and ErbB3 were unable to undergo phosphorylation when stimulated with EGF or NDF, respectively ((Beerli et al., 1995; Graus-Porta et al., 1995). This demonstrated that the lack of ErbB2 surface expression leads to inhibition of transphosphorylation and heterodimerization. Further studies support these finding by demonstrating that ErbB2 or ErbB3 singly expressed in cells devoid of other ErbBs cannot be activated by ErbB ligands, even at very high ligand concentrations. However, in the presence of a co-receptor, ErbB2 and ErbB3 promote strong intracellular signaling (Harari and Yarden, 2000; Pinkas-Kramarski et al., 1996; Pinkas-Kramarski et al., 1997). Taken together, these results suggested that ErbB2 may play an important role in the ability of the other EGFR members to induce transformation (Graus-Porta et al., 1997; Karunagaran et al., 1996; Tzahar et al., 1996).

1.7 SIGNALING FROM ERBB2/NEU TYROSINE KINASE

Receptor dimerization leads to kinase activation and phosphorylation of cytoplasmic tyrosine residues which serve as docking sites for a variety of cytoplasmic signaling molecules containing SH2 or PTB domains (Pawson, 1995). Analysis of the repertoire of signaling molecules that associate with ErbB2 revealed no unique substrate which would explain the transforming ability of this oncogene. However, the transforming mutant of ErbB2, which serves as a model system for human breast cancer, strongly interacts with both the phosphotidylinositol 3- kinase (PI-3K) pathways (Peles et al., 1992) and the mitogen activated protein kinase (MAPK) (Ben Levy et al., 1994). These observations support that cell proliferation and cell survival are activated by ErbB2 through these respective biochemical pathways.

The enzyme phospholipase C-γ (PLC-γ) was the first substrate shown to associate with ErbB2 by coimmunoprecipitation studies using NIH3T3 cells (Fazioli et al., 1991;

Peles et al., 1991). The following year, Jallal et al demonstrated the same interaction in human mammary tumor derived cell lines (Jallal et al., 1992). Phosphotidyl inositol kinases are additional substrates of ErbB2 that generate lipid second messengers. Scott et al have shown that activation of ErbB2 stimulates PI-3K activity in BT474 cells (Scott et al., 1991). In addition to PI-3K, ErbB2 can also regulate the Ras/MAPK pathway. The Ras-GTPase activating protein (Ras-GAP) SH2 domain can bind and activate Neu *in vitro* (Muthuswamy et al., 1994). In addition to PI-3K and Ras, c-Src and c-Yes are also able to associate with ErbB2 both *in vitro* and *in vivo* (Luttrell et al., 1994; Muthuswamy et al., 1994; Muthuswamy and Muller, 1995a; Muthuswamy and Muller, 1995b).

Adaptor molecules such as Grb-2 and Shc are also considered substrates of ErbB2 since they are involved in linking ErbB2 to the Ras/MAPK pathway. Grb-2 exists in a complex with the guanine nucleotide exchange factor Son of sevenless (Sos), which can activate Ras by catalyzing the exchange of bound GDP for GTP (McCormick, 1994). Grb-2 contains SH3 domains and proline-rich regions within the C-terminus of Sos. This allows the SH2 domain of Grb-2 to bind either Shc or ErbB2. Interestingly, many ErbB2-overexpressing breast cancer cells also overexpress Grb-2 (Daly et al., 1994). In fact studies have demonstrated physical complexes between ErbB2 and Grb-2 in several breast tumor-derived cell lines (Janes et al., 1994). The importance of Grb-2 in mammary tumorigenesis is substantiated by the fact that Grb-2 mutants that lack their SH3 domains decrease transformation driven by activated ErbB2 (Xie et al., 1995).

1.8 ERBB2 OVEREXPRESSION AND THE CELL CYCLE

The D-type cyclins are induced by numerous mitogenic stimuli and play a central role in initiation of cell cycle progression. Cyclin D1 has been shown to play an important role in mammary transformation since its aberrant expression can reduce or overcome the dependency of mitogenic stimulation (Bartkova et al., 1997; Sherr and Roberts, 1999; Weinberg, 1995). In fact, cyclin D1 is overexpressed in approximately 40% of human breast cancers (Bartkova et al., 1994). Its overexpression is particularly high (>75%) in ductal carcinomas *in situ* (DCIS), the majority of which also overexpress ErbB2 (Allred et al., 1992; Mack et al., 1997). Other cyclin genes are very rarely

amplified in breast cancer (Courjal et al., 1996; Gillett et al., 1994). ErbB-dependent mitogenic signaling has been shown to channel through the activation of cyclin D1. In particular, cell lines transfected with either wild type or oncogenic ErbB2 show a dramatic upregulation of cyclin D1 protein expression and this upregulation is at least in part initiated at the transcription level (Lee et al., 2000). Involvement of cyclin D1 in ErbB2-mediated tumorigenesis is further supported by various studies demonstrating that cyclin D1 can be upregulated by activated Ras, Raf, MEK and Rac, all downstream targets of ErbB2 signaling cascades (Albanese et al., 1995; Aziz et al., 1999; Cheng et al., 1998; Gjoerup et al., 1998). These data implicate the requirement for cyclin D1 in breast cancer cells overexpressing ErbB2.

Direct evidence demonstrating the importance of cyclin D1 during neu-mediated transformation derives from studies using cyclin D1 deficient mice (Bowe et al., 2002; Yu et al., 2001b). Yu et al., have shown that mice lacking cyclin D1 expression are resistant to breast cancers induced by neu and ras but remain sensitive to other oncogenic pathways such as c-myc. This suggests the depency of neu on cyclin D1 for malignant transformation.

1.9 SIGNALING FROM ERBB3 TYROSINE KINASE

ErbB3 is a unique member of the EGFR family of protein tyrosine kinases. As mentioned earlier, the protein tyrosine kinase activity intrinsic to ErbB receptors is critical to their function. However, unlike other ErbB family members, the ErbB3 receptor lacks intrinsic kinase activity (Guy et al., 1994b). Interestingly, this does not prevent ErbB3 from being phosphorylated on C-terminal tyrosine residues. Studies suggest that this is due to ligand-driven or ligand-independent heterdomeric interactions with other ErbB family members (Kim et al., 1994; Soltoff et al., 1994; Wallasch et al., 1995).

Of the various heterodimers formed, the ErbB2/ErbB3 dimer constitutes a co-receptor which is capable of eliciting potent mitogenic signaling (Carraway, III et al., 1995; Pinkas-Kramarski et al., 1996) as well as transforming signals (Cohen et al., 1996; Zhang et al., 1996). Indeed studies have shown that NIH3T3 fibroblasts devoid of

endogenous ErbB expression is non-transforming when transfected with ErbB2 alone. However, coexpression of ErbB3 enhances ErbB2-mediated transformation of NIH3T3 cells and Rat-1 fibroblasts (Alimandi et al., 1995; Siegel et al., 1999). This notion is further supported by using ErbB2-expressing transgenic mouse models (Siegel et al., 1999). Here Siegel et al show that sites of activated ErbB2-induced tumor formation show elevated levels of ErbB3 expression. Consistent with these results is the observation that ErbB2 and ErbB3 are frequently overexpressed in human breast cancer cells (Naidu et al., 1998; Siegel et al., 1999). These results highlight the importance of the ErbB2/ErbB3 heterodimer during mammary tumorigenesis and reflect the ability of this heterodimer to activate cellular signaling pathways that elicit cellular proliferation and transformation.

The carboxyl-terminus of ErbB3 contains several docking sites for signal-transducing proteins containing SH2,SH3 and PTB domains (Hellyer et al., 1998; Olayioye et al., 2000) and hence increasing the mitogenic signaling for ErbB3-containing heterodimers. ErbB3 contains one Asn-Pro-Xaa-Tyr (NPXY) and six Tyr-Xaa-Xaa-Met (YXXM) sequence motifs, which are consensus binding sites for Shc and PI-3K, respectively. Shc is also known to interact with EGFR and ErbB2 through its PTB domain (Batzer et al., 1994; Dankort et al., 2001; Dankort et al., 1997). Upon association, Shc activates the Ras/mitogen-activated protein kinase (MAPK) signaling cascade (Guan, 1994; Seger and Krebs, 1995) which is well known to regulate cell proliferation pathways (Pages et al., 1993; Posada and Cooper, 1992).

In addition to recruiting Shc, ErbB3 also recruits PI-3K (Kim et al., 1994; Soltoff et al., 1994). The PI-3K protein consists of two subunits namely p85 and p110 (Escobedo et al., 1991; Hiles et al., 1992; Hu et al., 1993). The p85 regulatory subunit of PI-3K contains SH2 domains which associate with the YXXM motifs of ErbB3 (Hellyer et al., 1998). The p110 catalytic subunit is required for the catalytic function of PI-3K (Dhand et al., 1994; Otsu et al., 1991). Each of the six YXXM motifs on ErbB3 has the ability to bind the PI-3K holoenzyme (Hellyer et al., 2001). The regulatory subunit maintains the catalytic unit in a low-activity state in quiescent cells and mediates its activation by direct interaction with phosphotyrosine residues (Otsu et al., 1991). Direct binding of p110 to activated Ras further stimulates PI-3K activity (Kodaki et al., 1994).

Activated PI-3K then phosphorylates phosphoinositides on the D-3 position of the inositol ring (Varticovski et al., 1994) in response to cell stimulation by growth factors. The phosphorylated lipid product phosphotidylinositol-(3,4,5)-trisphosphate (PIP_3), acts as a second messenger that mediate mitogenic and transforming signals within the cell (Toker and Cantley, 1997).

How does lipid phosphorylation set in motion a coordinated set of events leading to cell growth, cell cycle entry and cell survival? Many signaling proteins, including protein serine-threonine kinases and protein tyrosine kinases have pleckstrin homology domains that specifically bind to D-3 phosphorylated phosphoinositides ($PI(3,4,5)P_3$). These proteins are located in the cytosol of unstimulated cells and accumulate in the plasma membrane upon lipid phosphorylation. Of particular interest are the protein serine-threonine kinases Akt (also known as PKB) and phosphoinositide-dependent kinase 1 (PDK1). Association of these kinases with PIP3 at the membrane brings them into close proximity allowing phosphorylation of Akt by PDK1 (Lawlor and Alessi, 2001).

Phosphorylation of Akt stimulates its catalytic activity resulting in phosphorylation and activation of several other proteins and signaling cascades that affect cell growth and cell survival. Of all the known targets of Akt, the mammalian target of rapamycin (mTOR) is a particular interest of this thesis.

1.10 THE MAMMALIAN TARGET OF RAPAMYCIN (mTOR)

mTOR, also named FRAP (FK506-binding protein 12[FKBP12]- and rapamycin-associated protein), RAFT1 (rapamycin and FKBP-12 target-1) or RAPT1 (rapamycin target-1) is 289 kDa serine/threonie kinase (Sabatini et al., 1994; Brown et al., 1994; Sabers et al., 1995). Since the C-terminus of TOR is highly homologous to the catalytic domain of PI-3K, mTOR is a member of the phophoinositide 3-kinase-related kinase (PIKK) family. Studies show that Tor is a kinase that is highly conserved from yeast to humans. Human, mouse and rat mTOR proteins share 95% identity at the amino acid level (Abraham and Wiederrecht, 1996; Gingras et al., 2001b; Jacinto and Hall, 2003). mTOR consists of several domains and three of which are a catalytic kinase domain, a

FKBP12-rapamycin binding (FRB) and a putative auto-inhibitory domain near the C-terminus. Several biochemical and genetic studies suggest that FKBP12 is the most important binding protein with respect to the rapamycin-sensitive signal transduction pathway (Fruman et al., 1995; Heitman et al., 1991; Koltin et al., 1991).

The cellular localization of mTOR varies according to the cell line and culture conditions. In HEK293 cells, mTOR is distributed in the cytoplasm, most likely associated with the membrane of the endoplasmic reticulum (Drenan et al., 2004). In contrast to HEK293 cells, cellular immunostaining and fractionation studies reveal nuclear localization of mTOR in several tumor cell lines (Zhang et al., 2002). Indeed studies have demonstrated that cytoplasmic-nuclear shuttling might be important for mTOR function (Kim and Chen, 2000). Although a recent study suggests that cellular stress plays and important role in altering mTOR cellular distribution (Tirado et al., 2003), the exact reasons for mTOR shuttling remains unclear.

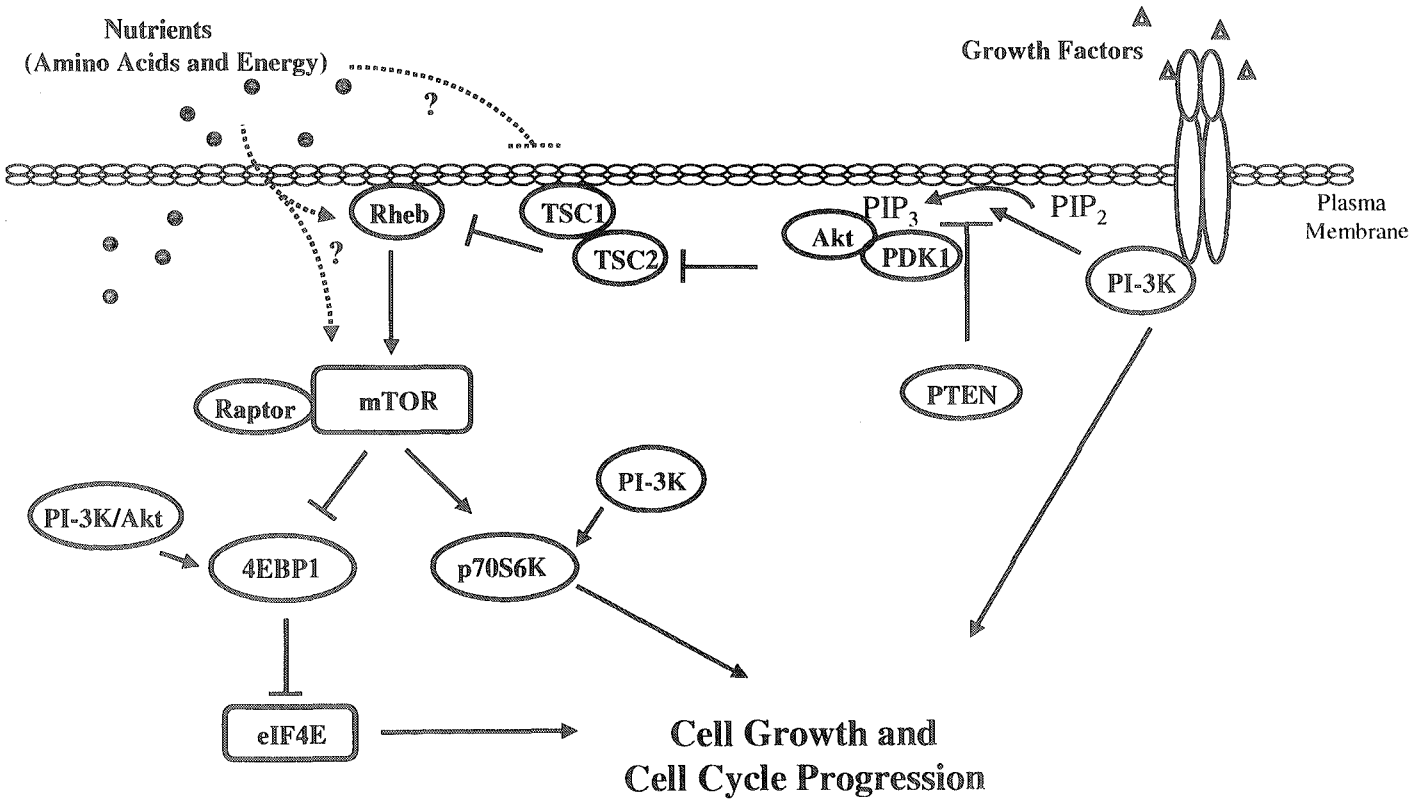
In mammalian cells, mTOR is regulated by availability of nutrients (amino acids and glucose) as well as growth factors (Fang et al., 2001; Jacinto and Hall, 2003; Peng et al., 2002; Proud, 2002). Studies suggest that growth factors regulate mTOR signaling directly through the PI-3K/Akt signaling pathway (Downward, 1998; Hu et al., 2000; Nave et al., 1999; Scott et al., 1998; Sekulic et al., 2000), however the mechanisms of how nutrients activate mTOR remain unknown. In response to growth stimuli, quiescent cells increase the translation of a subset of mRNAs whose protein products are required for traverse through the G1 phase of the cell cycle. There is accumulating evidence to suggest that mTOR is involved in the coupling of growth stimuli with cell cycle progression and therefore being the central regulator of cell growth and survival (reviewed in (Mita et al., 2003b; Raught et al., 2001).

1.11 mTOR SIGNALING

The importance of mTOR in the regulation of cell growth and proliferation has led to the discovery of several proteins (PI-3K, Akt, TSC1, TSC2, Rheb, Raptor, S6K, 4EBP1) involved in the mTOR signaling pathway (Figure 1.2).

Figure 1.2. The target of rapamycin signaling pathway. mTOR is regualated by PI-3K-dependent signaling and by nutrients. Growth factors activate receptors on the cell surface. These signal to PI-3K, which can be inhibited by PTEN. PI-3K in turn, activates phosphoinositide-dependent protein kinase 1 (PDK1) and Akt. Akt phosphorylates TSC2 and activates the TSC1/2 complex, which acts as a negative regulator of the small GTP-binding protein Rheb that might be the direct activator of mTOR. mTOR binds to raptor and either directly or indirectly regulates ribosomal protein p70S6K (S6K) which in turn regulates translation initiation. mTOR directly phosphorylates and inactivates eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4EBP1), the suppressor of eIF4E. Release of eIF4E from phosphorylated 4EBP1 leads to binding of eIF4E to the 5'untranslated region, which is required for cap-dependent translation of mRNAs. Amino acid sufficiency is sensed by TSC1/2 complex, Rheb and mTOR. Arrows depict activation, bars depict inhibition and dotted lines depict unknown pathways.

Figure 1.2


Nutrients
(Amino Acids and Energy)
?
Growth Factors
Rheb
TSC1
TSC2
PIP3
PIP2
Akt
PDK1
PI-3K
Plasma
Membrane
?
Raptor
mTOR
PTEN
PI-3K
PI-3K/Akt
4EBP1
p70S6K
eIF4E
Cell Growth and
Cell Cycle Progression

Upstream signaling pathways

PI-3K/Akt. An Akt consensus phosphorylation site, S2448, was shown to be phosphorylated on mTOR in vivo. Furthermore, growth factor stimulation leads to an increase in S2448 phosphorylation in a PI-3K/Akt dependent manner (Nave et al., 1999; Sekulic et al., 2000). On the contrary, a mutant mTOR possessing an alanine substitution at the S2448 site still retains its ability to phosphorylate and activate its downstream targets after growth factor stimulation (Sekulic et al., 2000). Furthermore, other studies have shown only a modest increase in mTOR kinase activity in mammalian cells overexpressing Akt suggesting that Akt may not be a major effector of mTOR. Due to conflicting results, it is unclear to what extent mTOR activity is regulated by PI-3K/Akt and whether or not the two signaling pathways function independently. Therefore, the role of the S2448 phosphorylation event in the regulation of mTOR currently remains unclear.

Mitogen activated Akt also associates with and inactivates the tuberous sclerosis (TSC) complex, formed by hamartin (TSC1) and tuberin (TSC2) (36), by phosphorylating TSC2 (Inoki et al., 2002; Potter et al., 2002). The TSC1/TSC2 complex inhibits TOR activation since loss of the complex results in an mTOR-dependent increase in the activity of mTOR-targets (Gao et al., 2002). However, whether this occurs through the binding of TSC complex to mTOR is unclear. Another phenomenon that is unclear is how phosphorylation of TSC2 regulates its function. One model proposes that Akt-mediated phosphorylation destabilizes TSC1/TSC2 interactions thereby inhibiting formation of the complex and activating mTOR kinase activity (Inoki et al., 2002; Potter et al., 2001). In contrast, others have shown that Akt-dependent phosphorylation of TSC2 was not sufficient to disrupt the TSC complex in mammalian cells (Dan et al., 2002; Manning et al., 2002), or to increase the turnover of overexpressed TSC2 (Tee et al., 2003). Several studies indicate that binding of 14-3-3 protein family members to phosphorylated TSC2 is essential in rapid degradation of TSC2 thereby providing another mechanism for inactivation of the TSC complex (Li et al., 2002b; Nellist et al., 2002).

How the TSC complex regulates mTOR signaling was clarified by the discovery that TSC2 negatively regulates a small G protein named Rheb (Ras homolog enriched in

brain). TSC2 acts as a GTPase-activating protein (GAP) and maintains Rheb in its inactive GDP-bound state which ultimately results in downregulation of mTOR. Many studies have shown that Rheb functions as a positive regulator of cell and organ growth localizing downstream of PI-3K/Akt and TSC1/2 but genetically upstream of mTOR and its translational targets (Inoki et al., 2003; Saucedo et al., 2003; Stocker et al., 2003).

Another protein that contributes to mTOR function is known as raptor (Hara et al., 2002). It is a 150 kDa protein that contains seven WD repeat motifs and seems to have dual function. It inhibits mTOR under nutrient-deficient conditions and stimulates mTOR in a nutrient-replete environment (Hara et al., 2002; Kim et al., 2002). Consistent with this model, inhibition of raptor expression with small interfering RNA reduces cell size and recapitulates the phenotypes observed during mTOR deficiency. It has also been suggested that raptor may act as a scaffold protein that links mTOR to its downstream translational regulators such as S6K and 4EBP1 (discussed below)(Hara et al., 2002).

Downstream signaling pathways

As mentioned earlier, activation of receptors coupled to PI-3K signaling has been implicated in the stimulation of a complex network of downstream effector molecules, including a number of kinases known to affect specific components of the translational apparatus. Indeed several studies have shown that TOR regulates translation in mammalian cells (reviewed in (Gingras et al., 1999b) by regulating the phosphorylation state of several different translational effector proteins such as p70 ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein 1 (4EBP1).

The ribosomal S6 kinase regulates the translation of a unique family of mRNAs that possess a 5' terminal oligopyrimidine tract (5'TOP). This tract of 4-14 pyrimidines is found at the 5' terminus of ribosomal protein mRNAs as well as mRNAs coding for other translational effectors. The 5'TOP family members make up 20-30% of the total cellular mRNA, including ribosomal proteins and translational elongation factors whose increased expression is essential for cell growth and proliferation. Although the mechanism of 5'TOP mRNA regulation is poorly understood, it is well understood that S6K plays an important role in the modulation of 5'TOP translation.

S6K was initially described as a kinase activity responsible for the phosphorylation of S6 isolated from mitogen-stimulated Swiss mouse 3T3 cells (Jeno et al., 1988). Subsequent purification, cloning and expression studies revealed that two isoforms of the kinase are produced from the same transcript (Banerjee et al., 1990; Grove et al., 1991; Kozma et al., 1990). The shorter form is largely cytoplasmic (p70S6K) whereas the larger form appears to be exclusively nuclear (p85S6K). With the recent identification of a highly homologous gene product, S6K2, p70S6K/p85S6K have been collectively termed S6K1 or generally referred to as S6K.

S6K contains a highly acidic amino terminus, followed by a typical serine/threonine kinase catalytic domain and a regulatory carboxy-terminal tail. Four serine-threonine phosphorylation sites have been identified in the carboxyl-terminal tail (Ferrari et al., 1992). All four phosphorylation sites exhibit a degree of phosphorylation in quiescent cells, which is then increased upon mitogen treatment.

Several upstream effectors of S6K have been identified such as PDK1, Akt, mTOR as well as amino acids. For purposes of the thesis, the focus will reside on the role of mTOR is S6K regulation.

Upon activation by proliferative stimuli mediated by PI-3K/Akt signal transduction pathway, mTOR phosphorylates and activates S6K which, in turn, phosphorylates the 40S ribosomal protein S6 (Hu et al., 2000). The phosphorylation of S6 protein allows recruitment of the small 40S subunit into actively translating polysomes. Studies have shown that mTOR phosphorylates S6K at T389 site which most closely parallels kinase activity of the protein (Pearson et al., 1995). Conversion of T389 to either acidic or neutral amino acids revealed that T389 was a critical regulatory site, whereas the other sites played a modulatory function (Dennis et al., 1996).

Several studies have also reported an alternative mechanism of S6K regulation by mTOR. It has been proposed that mTOR represses a serine-threonine phosphatase that dephosphorylates rapamycin-sensitive sites of S6K (Brown et al., 1995; Burnett et al., 1998)(Brown et al., 1995; Burnett et al., 1998). The reversal of repression of this phosphatase by inhibition of mTOR may be the explanation as to why several studies observe a rapid dephosphorylation of S6K upon treatment with rapamycin and stimulation with growth factors (Di Como and Arndt, 1996).

Regulation of protein translation is an important aspect of the control of cell growth. A rate-limiting step in this process is the binding of the mRNA to the small ribosomal subunit, a step mediated by the eukaryotic initiation factor 4 (eIF4) group of proteins. eIF4F, though its smaller subunit eIF4E recognizes the cap structure that is present at the 5' end of all cellular mRNAs (reviewed in (Gingras et al., 2001b).

Overexpression of eIF4E in rodent cells leads to cellular transformation and eIF4E has been implicated in cell cycle control (Lazaris-Karatzas et al., 1990). Furthermore, studies show that NIH 3T3 cells expressing eIF4E are refractory to apoptosis induced by serum deprivation suggesting a cell survival role of eIF4E (Polunovsky et al., 1996).

eIF4E is the target of a family of translational repressors called 4EBPs (eIF4E-Binding Proteins, also known as PHAS). These repressors bind to eIF4E in order to inhibit its interaction with eIF4G and further incorporation into the eIF4F complex. This leads to inhibition of cap-dependent translation (Sonenberg and Gingras, 1998). In support of this, Rousseau et al have shown that overexpression of 4EBP1 in cells transformed by eIF4E, Ha-v-ras or v-src partially reverts their transformed phenotypes (Rousseau et al., 1996). The binding of 4EBP1 to eIF4E is dependent on the phosphorylation status of 4EBP1. In the unphosphorylated state, that predominates in quiescent cells, 4EBP1 binds to eIF4E which inhibits its activity (Gingras et al., 1999b; Sonenberg and Gingras, 1998). In response to mitogen or other stimuli, 4EBP1 becomes phosphorylated which in turn decreases its binding affinity for eIF4E (Gingras et al., 2001b). These actions promote the dissociation of 4EBP1 from eIF4E thereby increasing the availability of eIF4E which can in turn bind to eIF4G, 4B and 4A, forming the multisubunit eIF4F complex. These interactions lead to increase in mRNA translation (Rosenwald, 1995; Sonenberg and Gingras, 1998). In support of this theory, studies have shown that growth factor deprivation and inhibition of mTOR results in dephosphorylation of 4EBP1 and an increase in eIF4E binding both of which lead to a decrease in translation of mRNAs required for cell cycle progression from G1 to S phase (Brunn et al., 1997; Gingras et al., 1998).

There is ample evidence indicating that activation of either PI-3K/Akt and mTOR is sufficient to induce phosphorylation of 4EBP1 on multiple sites (Fingar et al., 2002;

Gingras et al., 1998; Martin and Blenis, 2002). mTOR has been reported to phosphorylate 4EBP1 directly in vitro. However, it had not been clear until recently whether mTOR was responsible for the phosphorylation of all 4EBP1 sites, nor which sites must be phosphorylated in order to release 4EBP1 from eIF4E. Furthermore, the relative sensitivity of each site to different stimuli and pharmacological inhibitors also remains unknown. For example, some studies suggest that all of the phosphorylation sites are sensitive to serum and certain pharmacological agents (Fadden et al., 1997), whereas other studies observed a differential sensitivity for different subsets of sites (Gingras et al., 1998; von Manteuffel et al., 1997). In the following years, Gingras et al showed that phosphorylation of 4EBP1 by mTOR occurs on two sites only, Thr36 and Thr47 (Gingras et al., 1999a). Both these sites are phosphorylated to a very high degree in serum-starved cells allowing it to be active and complexed to eIF4E. Interestingly, reports have shown that phosphorylation of Thr37 and Thr46 by mTOR is not sufficient for 4EBP1 to dissociate with eIF4E. Further analysis indicate that these initial phosphorylation events only serve as priming events for subsequent phosphorylation of Ser65 and Thr70 which ultimately lead to dissociation of 4EBP1 from eIF4E and subsequent increase in mRNA translation.

Inhibition of mTOR in cells overexpressing PI-3K or Akt, blocks the phosphorylation of 4EBP1 suggesting that mTOR is required for these activities (Burgering and Coffer, 1995; Gingras et al., 1998; Li et al., 2002a).

1.12 MECHANISM OF ACTION OF RAPAMYCIN

As mentioned earlier, several highly regulated processes are aberrant in breast cancer leading to uncontrolled cell growth and proliferation. Several key proteins regulating these pathways may be strategic targets for therapeutic development against breast cancer. Indeed, several novel classes of therapeutics that interfere with specific elements of aberrant signaling and cell cycle regulation are being developed as anti-cancer agents (reviewed in Atalay et al., 2003; Hidalgo and Rowinsky, 2000; Mass, 2004). One such agent, rapamycin is being investigated as an anti-tumor agent by several different investigators (reviewed in (Houghton and Huang, 2004; Mita et al., 2003b;

Tolcher, 2004). The antiproliferative actions of rapamycin has been demonstrated due to its ability to modulate critical signal transduction pathways that link mitogenic stimuli to the synthesis of proteins required for cell cycle traverse from G1 to S phase (Wiederrecht et al., 1995).

Rapamycin is a macrolide fungicide isolated form the bacteria *Streptomyces hygroscopicus.* It binds intracellularly to members of the immunophilin family of FK506 binding proteins (FKBPs) and inhibits their enzymatic activity (Fruman et al., 1995; Heitman et al., 1991). Although there are several members of the FKBP family, biochemical and genetic studies suggest that FKBP12 is the most important binding protein with respect to the rapamycin-sensitive signal transduction pathway (Fruman et al., 1995; Koltin et al., 1991). The rapamycin-FKBP12 complex interacts with mTOR and blocks its kinase activity (Sabatini et al., 1994; Sabers et al., 1995). However, whether rapamycin-FKBP12 complex inhibits mTOR directly is an unresolved issue. Three possible models for the action of rapamycin have been proposed. First, FKBP12-rapamycin complex bound to mTOR destabilizes the mTOR-4EBP1/S6K scaffold complex which then leads to dephosphorylation of 4EBP1 and S6K (Hara et al., 2002; Kim et al., 2002). Since 4EBP1 and S6K regulate translation initiation, their dephosphorylation by rapamycin leads to inhibition of specific proteins and G1 arrest. Secondly, it has been shown that mTOR is a repressor of a cluster of protein phosphatases that regulate downstream targets. Binding of FKBP12-rapamcyin to mTOR leads to activation of certain protein phosphatases and the subsequent dephosphorylation of downstream targets such as 4EBP1/S6K (Gingras et al., 2001a; Oldham and Hafen, 2003). The third proposed mechanism of action of rapamycin is that FKBP12-rapamycin complex competes with a phosphatitic acid to bind to the FRB domain of mTOR and thereby blocking its kinase activity (Fang et al., 2001).

Other studies argue whether rapamycin really has a significant effect on mTOR activity. For example, studies have shown that rapamycin inhibits a moderate stimulation of mTOR kinase activity *in vitro* in response to stimuli and rapamycin can also inhibit mTOR autokinase activity *in vitro* (Scott et al., 1998)(Scott et al., 1998). However, other studies show that a much higher concentration of rapamycin is needed *in vivo* to elicit the same effect (Peterson et al., 2000). Other studies have shown very modest differences, or

no change at all in the kinase activity of mTOR after treatment with rapamycin (Peterson et al., 2000). Furthermore, rapamycin-treatment of cells in culture does not inhibit autophosphorylation at S2481. Therefore, although it is unclear whether rapamycin inhibits mTOR directly, it is clear that rapamycin functions through an inhibition of downstream signaling from mTOR and this repression may involve mechanisms other than direct repression of mTOR kinase activity.

Rapamycin has been shown to have a dramatic effect on both S6K and 4EBP1 phosphorylation. It exerts its inhibitory effects on S6K activation through either blocking activation of an upstream S6K kinase. Studies have identified rapamycin-sensitive phosphorylation sites on S6K and have shown that many of these sites were phosphorylated in response to mitogenic stimulation (Han et al., 1995). Furthermore, these sites were dephosphorylated upon rapamycin treatment in the hierarchical fashion Thr389>Ser404>Thr229 (Pearson et al., 1995). Mutational analysis revealed that the principal site of rapamycin-induced S6K inactivation was Thr389. This was further corroborated by the fact that substitution of an acidic residue in the Thr389 position led to an increase basal activity of the S6K that was extremely resistant to rapamycin treatment (Dennis et al., 1996; Pearson et al., 1995).

1.13 mTOR AS A TARGET FOR BREAST CANCER

Breast cancer provides one of the most well studied models that illustrate several different levels of pathway dysregulation. Since these aberrant signaling pathways distinguish between malignant and normal cells, they represent possible strategic targets for therapeutic developments against breast cancer. One such target, mTOR, was identified as a potential target for anticancer therapeutics after having seen that rapamycin demonstrated antineoplastic properties.

The anti-tumor activity of rapamycin was first discovered in the late 1970s, However, it was only until the mid-1990s that rapamycin re-emerged as a potential anti-cancer agent. In early studies, rapamycin was shown to inhibit the growth of a wide variety of murine and human cancers growing in both tissue culture and xenograft models in a concentration-dependent manner (Eng et al., 1984; Seufferlein and Rozengurt, 1996;

Wiederrecht et al., 1995). Many experimental cancers such as melanoma, leukemia, small-cell lung cancer, and B cell lymphomas demonstrated sensitivity to rapamycin in these studies. In most cases rapamycin induces G1 cell-cycle accumulation and arrest (Heitman et al., 1991). Rapamycin has also been shown to induce apoptosis of cells such as B cells, renal tubular cells and dendritic cells (Huang and Houghton, 2002). Studies have also shown that rapamycin induces p53-independent apoptosis in childhood rhabdomyosarcoma and enhances the apoptosis-inducing effects of cisplatin in murine leukemias and ovarian sarcoma *in vitro* (Hosoi et al., 1999; Shi et al., 1995). These experimental results serve as the impetus to further understand the mechanism by which rapamycin may be inhibiting tumor growth.

1.14 EXPERIMENTAL RATIONALE

1.14.1 BACKGROUND

A hallmark of the malignant phenotype is autonomous or unchecked cellular growth. Various components of the proliferative and survival pathways are constitutively activated or dysregulated in most human malignancies (Hanahan and Weinberg, 2000). Breast cancer, one of the most well studied models, illustrates many levels of pathway dysregulation. These levels include cell membrane receptors, hormone receptors, cytopasmic downstream circuitry, phosphatidylinositol 3-kinase and terminal elements that interact with nuclear targets (reviewed in (Atalay et al., 2003; Hidalgo and Rowinsky, 2000; Huang and Houghton, 2003; Mita et al., 2003a). Of relevance to this thesis is the observation that activating RTKs are amplified in the genesis of human breast cancer. In this regard, overexpression of wild-type ErbB2, generally attributable to gene amplification, occurs in 25-30% of human breast cancer and correlates with short time to relapse and lower overall survival (King et al., 1985; Slamon et al., 1987; Slamon et al., 1989; Yokota et al., 1986). Moreover, this poor prognosis correlates with enhanced catalytic activity of ErbB-2 (Bacus et al., 1996; DiGiovanna and Stern, 1995) suggesting that the constitutive activation of ErbB-2 regulated signaling pathways contribute to the progression of human breast cancers. The lipid kinase phosphotidylinoside 3-OH kinase

(PI-3K) and some of its downstream targets, such as the mammalian target of rapamycin (mTOR), p70 ribosomal S6 protein kinase (S6K) and the eukaryotic initiation factor 4E binding protein-1 (4E-BP1), are crucial effectors in oncogenic receptor tyrosine kinase signaling.

The generation of transgenic mice expressing wild type Neu (the rat homologue of ErbB2) in the mammary gland confirm the oncogenic potential of the Neu receptor in this tissue. Oncogenic mutations within the extracellular domain of the Neu receptor previously have been associated with mammary tumorigenesis in transgenic mice (Siegel et al., 1994; Siegel and Muller, 1996). However, studies have shown that the expression of altered Neu alleles is not sufficient to transform the entire mammary epithelium (Siegel et al., 1999).

One possible explanation is that in addition to Neu activation, the expression of another EGFR family member is required for efficient tumor induction. In this regard, Siegel et al. have shown that Neu-induced mammary tumors consistently express a dramatic 10 to 15-fold increase in endogenous ErbB3 protein levels without an increase in its transcript levels when compared to adjacent normal tissue suggesting a translational or post-translational regulation of ErbB3 during Neu-mediated tumorigenesis. This strong selective pressure for elevated levels of endogenous ErbB-3 protein highlights the importance of this receptor during Neu-mediated mammary gland transformation. Indeed, there is compelling evidence implicating that co-expression of both Neu and ErbB3 play a critical role in the induction of human breast tumors (Alimandi et al., 1995; Holbro et al., 2003; Pinkas-Kramarski et al., 1996; Siegel et al., 1999; Zhang et al., 1996).

1.14.2 PURPOSE

Although the ErbB-2/ErbB-3 heterodimer is capable of potent mitogenic signal, the role of ErbB3 in mammary tumorigenesis is not yet fully understood. The overall goal of this thesis is to elucidate the molecular pathway(s) involved in ErbB3 regulation and the biological significance of ErbB3 during Neu-mediated transformation.

1.14.2 HYPOTHESIS

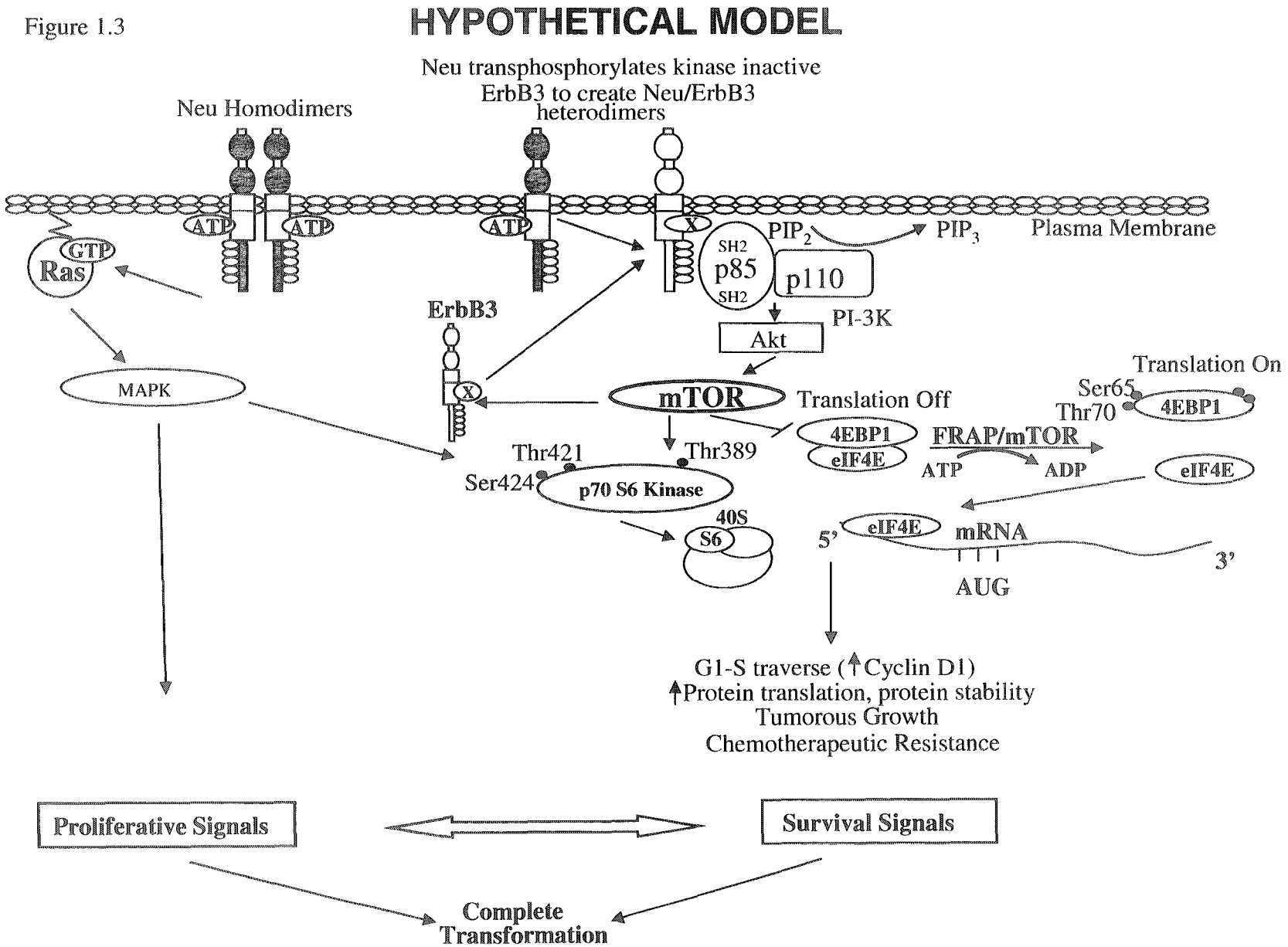
One potential explanation for Neu and ErbB3 co-expression is the recruitment of cooperative cell proliferative and cell survival signaling pathways during Neu-mediated mammary tumorigenesis. This hypothesis is illustrated in Figure 1.3. It has been previously shown that Neu induces transformation by sending strong proliferative signals predominantly through the Ras/MAPK pathway (Dankort et al., 1997). ErbB3, on the other hand, may be sending strong survival signals by recruiting p85 and activating the PI-3K/mTOR signaling pathway. Indeed, transgenic mouse models for breast cancer support the notion that both proliferative and survival signals must be activated within a cell before complete malignant transformation can occur (Amundadottir et al., 1996; Webster et al., 1998). Therefore, the overall hypothesis for this thesis is that Neu cannot act alone but requires ErbB3 for its full oncogenic potential. ErbB3 provides the necessary oncogenic signals by coupling to PI-3K and activating the PI-3K/mTOR pathway.

The details of the hypothetical model suggest: (1) Active Neu homodimers are necessary for induction of the proliferative Ras/MAPK signaling pathway. (2) Overexpressed Neu transphosphorylates kinase inactive ErbB3 which then recruits p85 and activates PI-3K/Akt and mTOR. (3) The ability of Neu to reach its full oncogenic potential is dependent on phospho-ErbB3-dependent recruitment and activation of PI-3K/mTOR cell survival pathway. (4) ErbB3-dependent activation of PI-3K leads to phosphorylation of S6K and 4EBP1. (5) Both PI-3K and mTOR regulate S6K and 4EBP1 phosphorylation and are involved in sustaining high levels of ErbB3 protein by increasing the translation and/or stability of ErbB3.

Cell proliferation and cell survival are distinct yet coupled processes that need to go hand in hand to induce transformation. Similar to cyclin dependent kinases (cdks), which are central regulators of cell proliferation, mTOR may be the central regulator of cell growth and survival. The PI-3K/Akt pathway has been implicated in the phosphorylation and activation of mTOR. This subsequently leads to increased phosphorylation of 4EBP1 and S6K, both of which are important steps in the stimulation of protein translation (Gingras et al., 1998; Gingras et al., 2001b). Indeed, studies have

Figure 1.3. Hypothetical Model Illustrating the potential signaling role of ErbB3 during Neu-mediated mammary tumorigenesis. This model illustrates the hypothesis of the thesis. Both Neu and ErbB3 function as an oncogenic unit to recruit proliferative and survival pathways, respectively. These pathways are distinct yet coupled and therefore cooperate to drive cell transformation. Briefly, Neu transphosphorylates and activates kinase inactive ErbB3 which then binds to the p85 regulatory unit of PI-3K and activates the PI-3K/mTOR pathway. This leads to phosphorylation of p70 S6 kinase and 4EBP1 which are required for translational and/or post-translational regulation of ErbB3 itself.

Figure 1.3


HYPOTHETICAL MODEL
Neu transphosphorylates kinase inactive ErbB3 to create Neu/ErbB3 heterodimers
Neu Homodimers
ATP
ATP
ATP
X
SH2
p85
SH2
p110
PIP2
PIP3
Plasma Membrane
GTP
Ras
ErbB3
PI-3K
Akt
MAPK
X
mTOR
Translation Off
Translation On
Ser65
Thr70
4EBP1
4EBP1
eIF4E
FRAP/mTOR
ATP
ADP
eIF4E
Thr421
Thr389
Ser424
p70 S6 Kinase
40S
S6
5'
eIF4E
mRNA
3'
AUG
G1-S traverse (↑Cyclin D1)
↑Protein translation, protein stability
Tumorous Growth
Chemotherapeutic Resistance
Proliferative Signals
Survival Signals
Complete Transformation

shown that overexpression of eIF4E leads to cellular transformation (Lazaris-Karatzas et al., 1990) and plays a role in cell survival (Polunovsky et al., 1996).

Since ErbB3 can bind and activate PI -3K directly (Fedi et al., 1994; Kim et al., 1994; Soltoff et al., 1994), ErbB3-dependent inactivation of 4E-BP1 and activation of S6K may, in part, account for elevated levels of ErbB3 protein in Neu-induced tumors. In support, studies have shown that PI-3K is activated by growth factor receptors and induces cell proliferation and cell survival. Indeed ErbB3 has been characterized as a major mediator of heregulin-dependent activation PI-3K pathway (Fedi et al., 1994; Soltoff et al., 1994; Wallasch et al., 1995). Recruitment and activation of PI-3K involves the binding of the SH2 domains of its p85 regulatory subunit to the phosphorylated YXXM motifs found in several signaling proteins (McGlade et al., 1992). ErbB3 is very well adapted to mediate PI3'K signaling since it contains six consensus p85 binding motifs in its C-terminal phosphorylation domain (Hellyer et al., 1998). Studies have shown that the phosphorylated motifs can directly associate with the SH2 domains of p85 (Hellyer et al., 1998; Prigent and Gullick, 1994). The significance of the recruitment and activation of PI-3K by ErbB3 during mammary tumorigenesis needs to be elucidated.

Aberrant signaling of the PI-3K/mTOR signaling pathways often distinguish malignant from normal cells and therefore may represent possible strategic targets for therapeutic developments. Furthermore, activation of the PI-3K/mTOR pathway has been correlated with chemotherapeutic resistance arguing that other treatment modalities may be necessary. Indeed, the mTOR inhibitor rapamycin, has demonstrated impressive antiproliferative actions in a diverse range of experimental tumors (Dilling et al., 1994; Eng et al., 1984; Guba et al., 2002; Mohi et al., 2004; Muthukkumar et al., 1995; Panwalkar et al., 2004; Seufferlein and Rozengurt, 1996; Yu et al., 2001a). Although the antitumor action of rapamycin has been attributed to its ability to modulate protein translation and G1 to S cell cycle phase traverse (Wiederrecht et al., 1995), the mechanism of how rapamycin inhibits tumor growth remains poorly understood. The rapamycin analog, CCI-779, is currently being evaluated in phase I/II clinical trials. Although it shows some promise as a potential therapeutic agent, a better understanding of the mechanism of mTOR regulation will identify those patients who may best respond to CCI-779 treatment. In this regard, the ability of rapamycin to inhibit Neu-mediated

tumor growth in transgenic mice was evaluated. The hypothesis is that inhibition of ErbB3 function and hence downregulation of the constitutively activated PI-3K/mTOR pathway may represent one mechanism by which rapamycin induces its anti-proliferative effects. This may represent one reason why ErbB2 overexpressing tumors are more likely to depend on rapamycin-sensitive pathways for growth and thus may be especially sensitive to rapamycin and its analogs.

1.14.4 TESTING THE HYPOTHESIS

In order to test the validity of the hypothetical model, various members of the Neu/ErbB3/PI-3K/Akt/mTOR signal transduction pathways were inhibited using either chemical inhibitors or dominant negative mutants (summarized in Figure 1.4). The signal transduction and biological effects of these inhibitions were then determined *in vivo* using tumor-transplanted and transgenic mouse models overexpressing Neu.

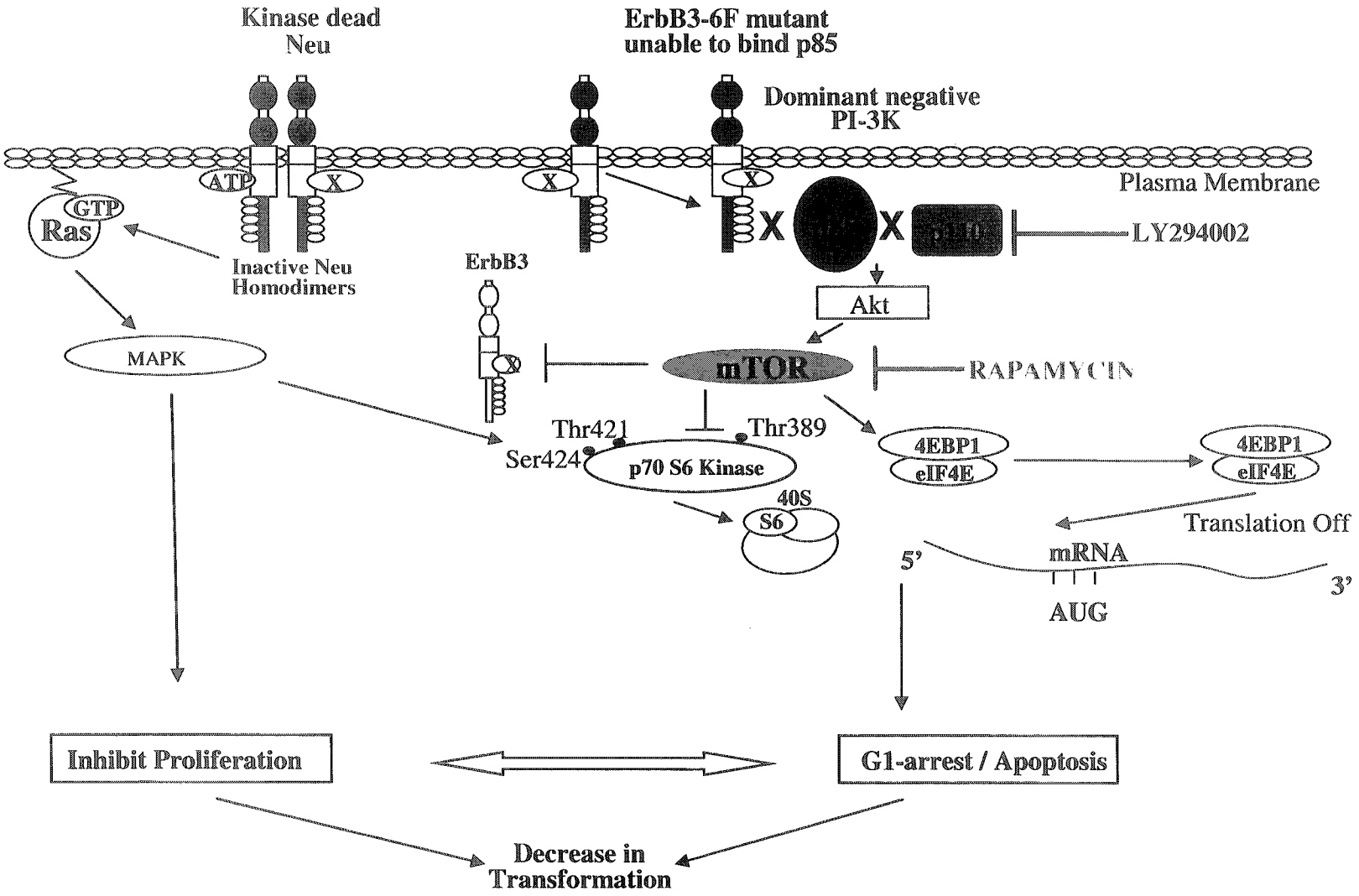
mTOR and PI-3K were chemically inhibited by the use of rapamcyin and LY294002, respectively. The effects of this inhibition on S6K, 4EBP1, cyclin D1, Neu and most importantly ErbB3 were determined. The observation that both mTOR and LY294002 led to a significant decrease in ErbB3 protein levels without affecting its transcript levels suggested a PI-3K/mTOR-dependent translational or post-translational regulation of ErbB3. Therefore, the effects of LY294002 and rapamycin on the stability of the ErbB3 protein were determined. Since the hypothesis suggests that PI-3K and mTOR predominantly send cell survival signals, the effects of rapamycin and LY294002 on cell cycle progression and apoptosis were also determined. Studies have suggested that LY294002 may not be a specific inhibitor of PI-3K. Rather, it may inhibit other members of the PIKK family. In order to ensure the effects of LY294002 were due to inhibition of PI-3K alone, a dominant negative mutant of PI-3K (Δp85) was introduced into a Neu-expressing cell line. The mutant contains alterations in its p85 regulatory domain that prevents it from binding and activating the p110 catalytic domain.

Neu activation was inhibited by use of an adenovirus expressing the Neu protein with an inactive kinase domain (NeuKD). The NeuKD (Neu Kinase Dead) receptor

Figure 1.4. Testing the hypothetical model by the use of chemical inhibitors and mutants. In order to test the validity of the hypothetical model, various members of the Neu/ErbB3/PI-3K/Akt/mTOR signal transduction pathways were inhibited using either chemical inhibitors or genetic mutants. Briefly, mTOR and PI-3K were chemically inhibited by the use of rapamcyin and LY294002, respectively. The dominant negative mutant of PI-3K (Δp85) contains alterations in its p85 regulatory domain that prevents it from binding to and activating the p110 catalytic domain. The NeuKD (Neu Kinase Dead) receptor retains its ability homo-or heterodimerize with Neu and ErbB3, respectively but loses its ability to auto- and transphosphorylate tyrosine residues in the carboxyl terminus. The ErbB3-6F mutant contains mutations in all six (YXXM) of its p85 docking sites that prevents it from interacting with and activating PI-3K. This allows for direct assessment of the importance of the ErbB3/p85 interaction. Finally, the signal transduction and biological effects mTOR and ErbB3 inhibition was also determined *in vivo* using tumor-transplanted and transgenic mouse models overexpressing Neu.

Figure 1.4

TESTING HYPOTHETICAL MODEL



retains its ability homo-or heterodimerize with Neu and ErbB3, respectively but loses its ability to auto- and transphosphorylate.

In order to determine the biological significance of the ErbB3/p85 interaction, a mutant ErbB3 (ErbB3-6F) was expressed in Neu-mediated tumor cells. This mutant ErbB3 contains mutations in all six (YXXM) of its p85 docking sites that prevents it from interacting with and activating PI-3K. This allows for direct assessment of the importance of ErbB3 in activating PI-3K and subsequently sending signals that govern mTOR-dependent cell cycle regulation as well as PI-3K-dependent antiapoptotic signals. It was also important to determine whether the ErbB3-6F mutant inhibits Neu-mediated transformation and induces apoptosis in Neu-mediated tumors since the hypothetical model suggests that these tumors highly depend on ErbB3's ability to recruit PI-3K in order to induce the survival signals necessary for complete transformation.

Studies have suggested that aberrant signaling of the PI-3K/mTOR signaling pathway distinguish malignant from normal cells and lead to chemotherapeutic resistance in many cases. Therefore, non-conventional therapies, such as the use of rapamycin and EGFR inhibitors alone or in combination have been under investigation. Although the rapamycin analog CCI-779 shows some promise as a potential therapeutic agent in Phase I/II clinical trials, a better understanding of the mechanism of mTOR regulation is important for targeting patients that will more favourably respond to this treatment.

In order to better understand the mechanism of action of rapamycin, randomized placebo controlled trials were conducted on mice transplanted with subcutaneous Neu-induced tumor cells as well as in transgenic mice overexpressing constitutively activated Neu in the mammary gland. Control groups received vehicle whereas treatment groups received intraperitoneal injections of rapamycin at various doses. Biochemical analyses of the tumors were performed in order to gain mechanistic insight into how rapamycin inhibits tumor growth.

To test growth inhibitory effects of AdErbB3-6F, Neu-mediated tumor cells were infected with AdErbB3-6F and transplanted into mice. Tumor volume was measured weekly for 4 weeks. For tumor regression studies using AdErbB3-6F, Neu-mediated tumor cells were transplanted subcutaneously into the flank of each mouse, and each group was randomized to intratumoral injections with AdErbB3-6F starting at either

week 1, week 2, week 3 or week 4 post-transplant. Two control groups were used (vehicle and Adlacz).

Figures presented in chapters 3 and 4 characterize various aspects of the hypothetical model.

CHAPTER 2

Materials and Methods

2.1 PLASMIDS

The pJ4Ω plasmid was kindly provided by Rowley and Bolen (Bristol-Myers Squibb). To construct the pJ4ΩErbB3 and pJ4ΩErbB3-6F expression plasmids, individual cDNAs were excised from pcDNA3B3X and pcDNA36F (Koland et al., 1999) shuttle vectors as HindIII- fragments and inserted into the corresponding sites on pJ4Ω. This placed the cDNAs encoding wild type ErbB3 and the mutant form under the control of the moloney murine leukemia virus (MoMuLV) promoter-enhancer. The pJ4ΩNeuNT and pJ4ΩNeu8142 plasmids were provided by Peter Siegel (McMaster University). The Δp85 construct was provided by Marc Webster (McMaster Universtity). The pJ4ΩRasV12 plasmid was provided by John Hassel (McMaster University). The pJ4ΩMT plasmid was constructed by excising the polyomavirus middle T antigen cDNA from MMTVMT (Guy et al., 1992a) as a Hind III-Eco RI fragment and ligating it into the cloning sites of pJ4Ω vector. A plasmid (PGK-puro) containing the puromycin resistant gene under the transcriptional control of phosphoglycerate kinase promoter was provided by Michael A. Rudnicki (McMaster University).

2.2 ADENOVIRUS CONSTRUCTION

2.2.1 Preparation of plasmid DNA

The vector expressing a kinase dead form of Neu (Ad NeuKD) was provided by Yongwon Wan (McMaster University) and was constructed as previously described (Chen et al., 2001). The Adlacz virus was also kindly provided by Frank Graham (McMaster Universty). The mutant ErbB3-6F receptor cDNA was provided by John Koland (University of Iowa) and was constructed with pcDNA-ErbB3 as a template by six sequential rounds of polymerase chain reaction mutagenesis (Stratagene) by which

each codon corresponding to Tyr-1051, Tyr-1194, Tyr-1219, Tyr-1257, Tyr-1273, and Tyr-1268 was substituted with phenylalanine codon. The ErbB3-6F Adenovirus was constructed as described in Ng and Graham (1995). First, the pDC116ErbB3-6F shuttle vector was generated using the pDNA36F plasmid. Briefly, the ErbB3-6F mutant cDNA was excised from pcDNA36F as a HindIII-HindIII fragment and was inserted into the pDC116 empty Ad shuttle plasmid. The plasmid was transformed into E.coli and grown in 500 mL of LB supplemented with 50 μg/ml ampicillin overnight at 37°C. The culture was transferred to a falcon tube and centrifuged at 6000g for 10 min at 4°C. The pellet was resuspended in 40 ml of cold solution I (10 mM EDTA, pH 8.0; 50 mM glucose, 25 mM Tris-HCl, pH 8.0) followed by 80 ml of freshly prepared solution II [1% sodium dodecyl sulfate (SDS); 0.2 N NaOH]. The resulting viscous lysate was allowed to sit for 10 minutes on ice after which it was resuspended in sterilized solution III (3M potassium acetate; 11.5% glacial acetic acid) and incubated for 20 minutes on ice. The tube was then centrifuged at 6000g for 10 minutes at 4°C and the supernatant was filtered using layers of cheesecloth. The plasmid DNA present in the supernatant was then precipitated by adding 0.6 volume of isopropanol and incubating for 30 minutes at room temperature. The plasmid DNA was pelleted by centrifugation at 6000g for 10 min at 4°C. The pellet was dried and dissolved in 5 mL of TE and 2 mL of pronase-SDS solution and incubated for 30 min at 37°C. CsCl (8.6g) was added to the DNA mix, incubated on ice for 30 min and centrifuged at a maximum speed in a Beckman tabletop centrifuge for 30 min at 5°C. The supernatant was slowly collected using a 10 mL syringe and 16-gage needle in order to avoid the pellicle. 25 μl of 10 mg/ml ethidium bromide was added and the supernatant was then transferred to a Beckman VTi 65.1 ultracentrifuge tube. The tube was sealed and centrifuged in a Beckman VTi 65.1 rottor at 55 000 rpm (288 000g) for 14 hours at 14°C. The supercoiled plasmid DNA band was collected using a 3 mL syringe and 18-gage needle. The plasmid DNA was then transferred to a 15 mL polypropylene tube containing 5 mL of isopropanol. The tube was mixed immediately to extract the ethidium bromide from the solvent layer. The phases were allowed to separate and pink ehtidium bromide-containing layer was removed. This process was repeated until the solvent layer was completely colourless. The volume was brought up to 4 mL with TE and then 8 mL of cold 95% ethanol was added and mixed by inversion in order to

precipitate the DNA . The tube was then centrifuged at top speed for 15 min in order to pellet the DNA. The pellet was washed twice in 70% ethanol, allowed to dry and resuspended in TE to a concentration of 1-2 μg/μl. The plasmid DNA concentration was determined by OD_{260}.

2.2.2 Maintenance of 293 cells

Low passage 293 cells were maintained in 150 mm tissue culture dishes and were split 1 to 3 when they reached approximately 90% confluency (every 2-3 days). Complete medium [minimum essential medium (MEM; Gibco-BRL) containing 10 % fetal bovine serum (FBS) (heat inactivated), 100 U/mL penicillin/streptomycin, 2mM L-glutamine, and 2.5 μg/mL fungizone] was used to maintain 293 cells. The medium was removed from the plates, the monolayer was rinsed twice with citric saline (135 mM KCl, 15 mM sodium citrate, autoclaved). The citric saline was removed from the plates and 0.5 mL citric saline was then added and left for 1 min. Cells were then resuspended in complete medium and distributed to new dishes. For cotransfections, a confluent 150 mm dish of 293 cells was split into eight 60 mm dishes for use the next day.

2.2.3 Adenovirus rescue by *in vivo* site-specific recombination

This step involves generating a large number of plaques by cotransfecting a single 60 mm dish of 293 cells with 2μg of shuttle plasmid and 2μg of Ad genomic plasmid. The shuttle plasmid (pDC116B3-6F) was generated as described in section....and pBHGloxE3Cre was used as the Ad genomic plasmid. Briefly, 293 cells were grown and allowed to reach 80% confluency in 60 mm dishes. One hour prior to cotransfection, the medium from the 60 mm dishes of 293 cells was replaced with 5 mL of freshly prepared complete medium without washing. Meanwhile, 8 mL of HEPES-buffered saline (HBS) and 40μl of salmon sperm DNA was combined in a conical tube and vortexed at maximum setting for 1 min. Two mL each of this solution was added to three polystyrene tubes labeled 'A', 'B' an d'C' and 1 mL into the fourth tube labeled 'D'. To tube 'A', 2 μg pDC116B3-6F shuttle plasmid and 2 μg of pBHGloxE3Cre genomic

plasmid was added. To tube 'B', 8 μg pDC116B3-6F and 8 μg of pBHGloxE3Cre was added. For tube 'C', 20 μg of pDC116B3-6F and 20 μg of pBHGloxE3Cre was added. These will ultimately result in four 60 mm dishes of 293 cells cotransfected with 0.5, 2 and 5 μg of each plasmid. Finally in tube 'D' 1 μg of the infectious Ad genomic plasmid pFG140 was added. This plasmid is used as control for transfection efficiency and under optimal conditions it should yield approximately 100 plaques per 0.5 μg of DNA. To tubes 'A', 'B', and 'C' 100 μl of 2.5 M $CaCl_2$ was added dropwise with gentle mixing and then incubated at room temperature for 30 min. Next, 0.5 mL of the contents of tube 'A' were applied to the monolayer of each of the 4 60 mm dishes. The same was done for the remainder tubes. The next day, the medium was removed from the dishes and overlayed with 10 mL of 0.5% agarose in 2× maintenance medium [2× MEM (Gibco-BRL) supplemented with 10% horse serum (heat inactivated), 200 U/mL penicillin/streptomycin, 4 mM L-glutamine, 5 μg/mL fungizone and 0.2% yeast extract] and allowed to solidify at room temperature after which they were returned to the incubator. Plaques began to appear within a week post-cotransfection. After 10 days, well-isolated plaques were picked from the monolayer by punching out agar plugs using a sterile cotton-plugged Pasteur pipet. The agar plugs were transferred into 0.5 mL PBS^{++} supplemented with glycerol to 10%, vortexed briefly and stored at -70°C.

2.2.4 Analysis of recombinant vectors

Once the plaques were isolated, they were expanded for extraction of AdErbB3-6F for analysis and to yield a working Ad vector stock. Briefly, 60 mm dishes of 293 cells (one per plaque) were seeded to reach 90% confluency. The virus plaque pick (0.2 mL) was added to the dish and allowed to absorb for 1 hr at 37°C after which 5 mL of maintenance medium was added and the dishes were returned back to the 37°C incubator until complete cytopathic effect (CPE) was observed (3-4 days post-infection). After complete CPE, the medium was supplemented with 10% glycerol and stored at -70°C. The rounded cells were treated with 0.5 mL pronase-SDS and incubated at 37°C for 4 hours. The lysate was transferred to microfuge tube containing 1 mL of 95% ethanol. The DNA was precipitated and centrifuged to form a pellet which was then washed with 70%

ethanol. The DNA was allowed to dry and resuspended in 50 µl of TE and subsequently dissolved by heating at 65°C. The structure of the AdErbB3-6F recombinant virus was analyzed by digesting 5 µl with Hind III.

2.2.5 Titration of Adenovirus

After verification of the DNA structure of AdErbB3-6F, it was purified and the concentration was determined by titration. 293 cells were seeded in 60 mm dishes and allowed to reach 90% confluency in 1-2 days. Serial dilutions of the AdErbB-6F recombinant virus were prepared (10^{-2}-10^{-6}) and 0.2 mL was used to infect each 60 mm dish. The dishes were incubated for 1 hour at 37°C, overlayed with 10 mL of 0.5% agarose in 2×maintenance medium, allowed to solidify and subsequently incubated at 37°C. Plaques were counted at 12 days post infection. The following formula was used to determine the titer of the adenovirus in pfu/mL: (number of plaques)(dilution factor)/(infection volume).

2.3 TISSUE CULTURE

2.3.1 Cell lines

All cell culture reagents were purchased from Gibco-BRL (Grand Island, NY, USA). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml) and fungizone (1 µg/ml). The NAFA cell line was provided by Dr. William Muller and was derived from tumors of transgenic mice overexpressing the point activated version of Neu in the mammary gland. Rat-1 fibroblasts were obtained from the American Tissue Type Collection (Rockville, Maryland). The Δp85NAFA stable cell line was derived by electroporation and selection with neomycin. Briefly, NAFA cells were transfected with a vector expressing the dominant negative PI-3K along with the neomycin-resistant gene. Cells positive for Δp85 gene were screened by treating the cells with mg/ml of G418 (neomycin). After the formation of colonies (10 days), the colonies were picked and

expanded into 24-well plates. After reaching confluence, each well was split 1 in 2 and. The first half was used to confirm positive colonies by detecting the presence of the neomycin-resistant gene using Southern blot analysis. Genomic DNA was extracted from the cells and were subjected to southern blot analysis using a neomycin-specific probe.

2.3.2 Focus Assays.

DNA was introduced into Rat-1 cells at a ratio of 40:1 of expression plasmid :puromycin resistant plasmid (PGK-puro) and colonies were selected in puromycin (3 μg/ml) containing media. Cotransfection of plasmids was conducted by lipofectamine trasfection (Gibco-BRL) following the manufacturer's instructions. For each cotransfection, one μg of each pJ4Ω expression plasmid was used and sheared salmon sperm DNA was used in the case where a single plasmid was transfected in order to ensure that equal DNA (2 μg) was used for each condition. Prior to transfection, 2×10^5 cells/well were seeded into 6-well plates. Post transfection, the cells were allowed to reach confluence and were then split into two 100 mm plates. The cells were maintained in 2% FBS in DMEM for 2 weeks after reaching a monolayer. The medium was changed every 3 days and at the end of 2 weeks the cells were washed with 1×PBS (140 mM NaCl. 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) and fixed for 4 hours with 10% buffered formalin phosphate and stained overnight with Geimsa stain (Fisher Scientific) following manufacturer's instructions. In the case of the NeuKD experiment, Rat-1 fibroblasts were infected with AdNeuKD (70pfu/ml) on day 2 post-transfection.

2.4 DNA ANALYSIS

2.4.1 Genomic DNA extraction from cell culture and mouse tissue

DNA from Δp85NAFA cells were extracted to determine neomycin-positive cells as well as to determine the time-course for excision of the Lox/Neo cassette upon Cre infection. Briefly, cells were treated with lysis buffer. Protein and RNA were digested with

proteinase K and RNase, respectively. The residual protein was removed by centrifugation and the DNA from the supernatant was recovered by precipitation with isopropanol. The DNA was washed 2× in 70% ethanol and resuspended in 100 µl of distilled H_20. To identify NDL2-5 transgenic progeny, genomic DNA was extracted from the tails of 4 week old pups. Approximately 1 cm of the tail was clipped and incubated overnight at 50°C in 500 µl of tail digestion buffer (10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 10 mM EDTA; 0.5% sodium dodecyl sulfate; 0.2 mg/ml proteinase K). The next day, the samples were extracted once with an equal volume of phenol-chloroform following by a single extraction with chloroform alone. The DNA was then precipitated with 100% ethanol, the pellet was dried and resuspended in 100 µl of distilled H_20.

2.4.2 Analysis of DNA by southern blot

To determine the time-course for excision of the Lox/Neo cassette upon Cre infection of Δp85NAFA cells, 15 µl of the genomic DNA solution was digested with 30 U of PvuII restriction enzyme at 37°C overnight. The digests were separated by electrophoresis through a 1% agarose gel and transferred to Gene Screen filters by Southern blot capillary action (Southern 1975). Briefly, the DNA fragments were denatured by incubating gel in denaturing solution (0.4 N NaOH; 0.6 M NaCl) for 45 min after which it was submerged in neutralizing solution (0.5 M Tris-HCl. pH 8.0; 1.5 M NaCl). The gel was then equilibrated in 20× SSC (1.5 M NaCl; 0.15 M sodium citrate) and for 45 min and place on a long piece of blotting paper (3 MM whatman) whose ends were suspended in a reservoir of 20× SSC solution. Gene Screen membrane was soaked in 20× SSC solution and placed on top of the gel, followed by additional whatman paper and stack of dry paper towels. The transfer occurred overnight after which the membrane is rinsed in 2× SSC. The single-stranded DNA molecules were then fixed to the membrane using an auto crosslinker (Stratalinker 1800, Stratagene). The probe was created by digesting the pJ4nlΔp85 plasmid with KpnI and PvuII to create a 650 bp fragment of Δp85. The probe was radiolabeled [α^{32}P]dCTP by random priming as described previously (Feinberg and Vogelstein, 1983). Briefly, 200 ng of DNA template was denatured in total volume of

7µl at 100°C for 5 min and then chilled on ice for 5 min. The following components were added to the DNA template: 2 µl of 10× cocktail C (0.5 M Tris-HCl, pH 8.0; 0.05 M $MgCl_2$; 0.5 mM 2-mercaptoethanol; 0.1 M each of dGTP, dATP, dTTP), 5 µl synthetic random oligonucleotide primers (hexamers, 2 µg/µl), 5 µl of [α^{32}P]dCTP (10 µci/µl, 3000 µCi/mmol), 10 U of Klenow fragment of DNA polymerase I. The template was incubated for 45 min at 37°C and then 230 µl of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) was added and the entire reaction was denatured for 5 min at 100°C. The membrane as incubated at 60°C for several hours in prehybridization solution (0.25 M Na_2HPO_4; 0.25 M H_3PO_4; 15% formamide; 7% SDS; 1% bovine serum albumin (BSA, Sigma) after which the denatured probe was added. The DNA and probe were allowed to hybridize overnight at 60°C. The membrane was then washed for 15 min at room temp followed by two 30 min washes at 60°C in 0.15 M sodium phosphate and 1% SDS. The membranes exposed to X-ray film overnight at -80°C.

2.4.3 Genotyping by PCR analysis

The genotypes of the potential NDL2-5 transgenic mice were determined by PCR using oligonucleotide primers that amplify a region of neu encoding the transmembrane domain and a small portion of the extracellular domain (nucleotides 1492 to 2116). The sequence of the primers used for PCR amplification were as follows: CGGAACCCAGATCAG-GCC and TTTCCTGCAGCAGCCTACGC. The PCR reaction was set up using PCR eppendorf tubes containing the following: 1 µl each of the oligonucleotide primers (10 µM), 2.5 µl 10X PCR buffer, 1 µl MgCl2 (50 mM), 1.5 µl deoxyribonucleotide triphosphate (dNTP) mix (5 mM each dATP, dCTP, dGTP, dTTP), o.2 µl of Taq DNA polymerase (5 units/ml), 16.8 µl of dH2O and 1 µl of genomic tail solution. The amplification was performed for 30 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min. A 1% agarose gel was used to resolve the PCR products. The gel was visualize by ethidium bromide staining.

2.5 RNA ANALYSIS.

2.5.1 RNA isolation

RNA from tissue culture cells and mammary tissues was isolated using guanidinium thiocyanate extraction followed by cesium chloride sedimentation gradient centrifugation (Chirgwin et al., 1979). Mammary tissue was homogenized in 3 mL of guanidium thiocyanate (GIBCO-BRL) solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol). The homogenized tissue was placed in a tube over 4 mL of cesium chloride (5.7 M cesium chloride containing 25 mM sodium acetate, pH 5.2). The RNA was pelleted by centrifugation at 32 000 r.p.m (20°C) using a SW41Ti rotor (Beckman) for at least 18 hours. The resulting pellet was resuspended in 100 µl of tissue resuspension buffer [5 mM EDTA (pH 8), 0.5% N-lauroylsarcosine, 0.7 M 2-mercaptoethanol] and 300 µl of diethyl pyrocarbonate (DEPC) treated water. The RNA was extracted by phenol-chloroform followed by precipitation with ethanol. Finally, the RAN yield was determined by U.V. absorption at 260 nm (1 OD_{260}=40µg/mL of RNA).

2.5.2 Plasmid templates for riboprobe synthesis

The antisense murine ErbB3 riboprobe was provided by Peter Siegel (McMaster University). The plasmid used to generate this riboprobe was constructed by inserting a 375 bp PCR product (corresponding to nucleotides 2489 to 2864 of the human ErbB3 cDNA) (Plowman et al., 1990) into the Eco RI-Bam HI sites of pSL301. This region of ErbB3 was PCR-amplified following a RT reaction with AB10860 (CGGAATTCGCAGCCTGGACCATGCCC) and AB10861 (CGGGATCCGTGTATT-TCCC-AAAGTGG) using RNA derived from an MMTV/NDL mammary tumor. The riboprobe was produced by digesting the template plasmid with Eco RI and transcribing the purified fragment with T7 RNA polymerase.

2.5.3 Synthesis of Riboprobe using *in vitro* transcription reaction

Linearized template plasmids were used in order to synthesize antisense ribonucleotide protection probes. The plasmids were digested and electrophoresed through a 1% gel and the fragment of interest was recovered from the gel by a gene using sodium iodide and glass milk beads (gene clean protocol provided by manufacturer, Geneclean, Biocan). A 25 µl *in vitro* transcription reaction (200 mM Tris-HCl, pH 7.5; 30 mM $MgCl_2$; 20 mM spermidineHCl; 8mM DTT; 40 U RNasin (Pharmacia); 100 µCi [α-^{32}P]UTP (10 µCi/µl, 3000 mCi/mmol); o.4 mM each GTP, ATP, CTP, and 0.04 mM UTP) required the use of 500 ng of DNA. The appropriate RNA polymerase (SP6, T3 or T7) was added (30 U) and allowed to incubate at 37°C for 45 min. An additional 30-60U of RNA polymerase was added to the reaction and further 30 min incubation at 37°C. The DNA template present in the reaction was removed by adding 20 U of RNase free DNase I, 1 µl $MgCl_2$ (0.5M), 20 µl of dH_20 and a 10 min incubation at 37°C. Next, dH_2O was added to bring the reaction volume to 100 µl after which an equal volume of phenol-chloroform was added and extraction of the aqueous phase took place. The labeled riboprobe was precipitated using 95% ethanol, 300 mM of sodium acetate (pH 5.2) and 20 µg RNase-free tRNA (Boehringer Manheim). The pellet was dried and resuspended in 100 µl of dH_20.

2.5.4 RNase protection analysis

RNase protection assays were performed in order to determine the levels of mRNA. The procedure was followed as described by Melton et al. (1994). Briefly, 20 µg of total RNA was mixed and incubated with [α-^{32}P]UTP-labeled antisense riboprobes at 85°C for 5 min in hybridization buffer (50% formamide, 400 mM NaCl, 40 mM piperazine-M-N'-bis (2-ethanesulfonic acid) pH 6.4 (PIPES), 1 mM EDTA pH 8.0). The reaction was incubated overnight at 50°C in order for hybridization to occur. The following day, the hybridization reactions were incubated with 300 µl of digestion buffer (300 mM NaCl; 10 mM Tris pH 7.4; 5 mM EDTA pH 8.0; 200 U RNase T1; 30 µl/mL RNase A) for 30 min at 37°C. The samples were further incubated with 20 µl of 10% SDS and 10 µl of proteinase K (10 µg/) for 20 min at 37°C. The samples were extracted using phenol-

chloroform and they were ethanol precipitated with 20 µg of RNase-free tRNA. The RNA pellets were then dried and resuspended in 10 µl of formamide loading buffer (80% formamide; 10 mM EDTA pH 8.0; 1 mg/ml xylene cyanol; 1 mg/ml bromophenol blue). The loading buffer containing the RNA was then heated to 95°C for 10 min and resolved on a 6% polyacrylamide/8M urea sequencing gel which was subsequently dried and exposed to Kodak XAR film at -80°C.

2.6 PROTEIN ANALYSIS

2.6.1 Preparation of protein extract from cell culture and tissue

Lysates from cell lines were prepared by first washing them 3 times in ice-cold PBS and then lysing for 20 min on ice in TNE lysis buffer (50 mM Tris pH 8; 150 mM NaCl; 1% NP-40; 2mM EDTA pH 8; 1mM sodium orthovanadate; 10 mM sodium fluoride; 10 µg/ml aprotonin; 10 µg/ml leupeptin). Proteins were extracted by centrifugation at 12 000g for 15 min at 4°C and the protein concentration was determined using the Bradford assay (Bio-Rad). Lysates from tissue samples were established by first grounding the tissue to a powder in liquid nitrogen followed by lysing for 20 min on ice in TNE lysis buffer. All lysates were cleared by centrifugation for 20 min and protein concentrations were determined by Bradford assay (Bio-Rad).

2.6.2 Immunoprecipitation

The desired proteins were immunoprecipitated by incubating the appropriate antigen-specific antibody, 20 µl of protein G-Sepharose beads (Pharmacia), and anywhere from 600-2000 µg of the protein lysate (depending on the desired protein) to a final volume of 600 µl for 3-5 hours at 4°C on a eppendorf rotator. ErbB3 immunoprecipitates were carried out using the C-17 antibody (2 µg/mL total lysate) by using 2 mg of total protein from lysates prepared as mentioned above. Immunoprecipitations for phosphor-tyrosine proteins were performed using PY-20 antibodies (2 µg/mg total lysate).

2.6.3 Immunoblotting

Lysates from cell lines were prepared by first washing them 3 times in ice-cold PBS and then lysing for 20 min on ice in TNE lysis buffer (50 mM Tris pH 8; 150 mM NaCl; 1% NP-40; 2mM EDTA pH 8; 1mM sodium orthovanadate; 10 mM sodium fluoride; 10 µg/ml aprotonin; 10 µg/ml leupeptin). Proteins were extracted by centrifugation at 12 000g for 15 min at 4°C and the protein concentration was determined using the Bradford assay (Bio-Rad). Lysates from tissue samples were established by first grounding the tissue to a powder in liquid nitrogen followed by lysing for 20 min on ice in TNE lysis buffer. All lysates were cleared by centrifugation for 20 min and protein concentrations were determined by Bradford assay (Bio-Rad). Immunoblots were performed using 20-100 µg of protein lysate (depending on origin on the lysate). Samples were resuspended in SDS gel loading buffer (62.5 mM Tris-HCl, 2% SDS, 5% glycerol, 0.8M 2-mercaptoethanol, 0.25% bromophenol blue), resolved by SDS polyacrylamide gel electrophoresis and transferred to Immobilon membranes (Millipore). Membranes were blocked for 1 hour in 5% (w/v) dry milk, TBS, and 0.1% Tween 20. Primary antibodies as indicated in figure legends were incubated with membranes overnight at 4°C and were then washed in TBS with 0.1% Tween 20. Subsequently, horseradish peroxidase-conjugated secondary antibodies were added and incubated for 1 hr at room temperature. The membranes were again washed in TBS with 0.1% Tween 20 and the membrane-bound proteins were visualized using an enhanced chemiluminescence detection system (Amersham).

2.6.4 Pulse Chase Analysis.

The half-life of ErbB3 after treatment of NAFA cells with rapamycin and LY294002 was determined using pulse chase analysis. Briefly, cells were plated at 80% confluence, washed in PBS and starved for 30 min at 37°C by adding 1 ml of DMEM (met/cys-free)/5% dialyzed FCS. Following starvation, 50 µl of Tran ^{35}S-label (500 µCi/plate) was added to the plates and incubated for 40 min at 37°C. Radioactive media was then removed and 5 ml of DMEM (FCS, 2mM methionine, 2mM cysteine) was added to all

plates. NAFA cells were then treated with either DMSO (control), rapamycin or LY294002 and allowed to incubate for 48 hrs. They were then washed in PBS, lysed in TNE lysis buffer, immunoprecipitated for ErbB3 and finally they were separated on a 12% SDS-PAGE gel electrophoresis. The gel was then transferred onto Immobilon P membrane and ^{35}S-labeled ErbB3 was quantified on a phosphoimager according to manufacturer's directions.

2.7 FLOW CYTOMETRY.

NAFA cells were treated with various concentrations of rapamycin for 24 hrs after which the cells were washed with PBS, trypsinized with trypsin-EDTA and resuspended in 1 ml of PBS. The cells were slowly fixed by adding ice-cold ethanol while gently vortexing the sample. The DNA content of each sample (approximately 2×10^6 cells) was estimated by staining with propidium iodide and then filtering the samples and quantifying the fluorescence by the use of a Cell Quest FACScan flow cytometer (Becton, Dickinson) according to manufacturer's directions.

2.8 APOPTOSIS ASSAY.

TUNEL was performed to detect apoptotic cell death. The adherent cultured cells were fixed in 1% paraformaldehyde in PBS (pH 7.4), washed with PBS and post-fixed in Ethanol:acetic acid at -20°C. Cells were then quenched in 3% H_2O_2 and subsequently incubated in TdT Enzyme for 1 hour at 37°C. The cells were then incubated with anti-digoxigen peroxidase conjugate for 30 min in a humidified chamber after which they were washed 4 times in PBS and stained for 3 min with peroxidase substrate. The cells were then counterstained in 0.5% (w:v) methyl green and washed 3 times with 100% n-butanol and dehydrated by washing in xylene. The cells were then mounted using Permount and viewed under the microscope.

2.9 TUMOR INDUCTION AND DRUG TREATMENT

Randomized placebo controlled trials were conducted on 8 week old female Fvb/n mice (Taconic Farms, Germantown, New York) transplanted with subcutaneous breast cancer cells (NAFA). In all trials groups of five Fvb/n mice were transplanted subcutaneously with 10^7 NAFA cells. Two, four-arm parallel group was randomized to treatment with placebo, 7.5, 10 or 15 mg / week of intraperitoneal rapamycin. Treatment with rapamycin began one day after NAFA cell transplantation in the first trial, and ten days after transplantation in the second trial. In both trials subcutaneous tumor volume was assessed with calipers 4 weeks after transplantation.

2.9.1 Treatment with Rapamycin.

In both NAFA transplanted trials four groups of five Fvb/n mice were transplanted with 10^7 NAFA cells subcutaneously in the right flank, and each group was randomized to treatment with vehicle, 7.5, 10 or 15 mg /kg/ week of intraperitoneal rapamycin. Treatment with rapamycin began one day after NAFA cell transplantation in the first trial, and ten days after transplantation in the second trial. Tumor size was measured on day 24 of study. Transgenic mice overexpressing constitutively activated Neu (Neu DeLetions in the extracellular domain) in the mammary gland (NDL2-5) were treated with rapamycin starting at 140 days of age (prior to palpable tumor onset). Both control and treatment groups consisted of 5 mice each. Control group received vehicle whereas treatment group received intraperitoneal rapamycin at 5 mg/kg twice weekly for a total of 8 weeks. At end of study, whole mammary gland (with or without tumor) was removed and used for biochemical analysis.

2.9.2 Adenoviral infection and intratumoral administration.

To test growth inhibitory effects of AdErbB3-6F, NAFA cells were infected at a multiplicity of infection (MOI) of 20 with either control adenovirus (Adlacz) or AdErbB3-6F. Two days after infection, cells were harvested, washed 3 times in PBS and injected subcutaneously into 8 week female Fvb mice. Tumor volume was measured

weekly for 4 weeks. For tumor regression studies using AdErbB3-6F, 10^7 NAFA cells were transplanted subcutaneously into the right flank of each mouse, and each group was randomized to intratumoral treatment with AdErbB3-6F starting at either week 1, week 2, week 3 or week 4 post-transplant. Two control groups were used (vehicle and Adlacz). Each mouse was injected intratumorally with the recombinant AdErbB3-6F.

2.10 HISTOLOGICAL EVALUATION

2.10.1 Autopsy of tumor-transplanted Fvb and NDL2-5 transgenic mice

For both transplanted Fvb and NDL2-5 transgenic mouse tumor model studies, the growth of transplanted tumors and of mammary tumors were monitored by regular physical palpation of the tumor transplantation site and of each mammary gland. Tumor bearing mice were sacrificed for autopsy by CO_2 asphyxiation when the tumor size had reached approximately 10% of body mass. Mice were considered tumor free if tumors failed to develop by the end of the treatment study. During autopsy, tumor tissues were fixed overnight at 4°C in 4% paraformaldehyde and transferred to 70% ethanol the following day. The Department of Anatomical Pathology at McMaster University kindly performed all the embedding, sectioning (5μm sections), and staining (hematoxylin and eosin). The histological analysis and description was kindly provided by Dr. Robert Cardiff (University of California, Davis, California).

2.10.2 Wholemount Preparation

Wholemount analyses were performed as described (Vonderhaar and Greco, 1979). Briefly, mice were sacrificed by CO_2 asphyxiation and the mice were placed on the dissecting board with their ventral side up. Their skin was washed with 70% ethanol and a midline incision was made through the skin, initiating at the tail end and terminating at the lower jaw. Further incisions were made extending from the midline incisions to the end of each paw. The skin was pulled away from the body wall using the flat ends of forceps and was pinned on the dissecting board. The number three thoracic mammary

gland from either side was dissected out by initiating the cut site from the inner surface of the skin using scissors. The excised glands were quickly spread onto glass slides and allowed to dry overnight in acetone. The glands were then allowed to stain overnight in Harris' modified hematoxylin. The next day, the glands were destained by washing it in destain solution (1% HCL in 75% ethanol). This process was repeated until the epithelial component was seen in sharp contrast to the light background of the fat pad. The glands were then placed for one minute in 0.002% ammonium hydroxide in order to fix the stain and were then transferred to 70% ethanol for a few hours followed by 100% ethanol. Finally, the gland was placed in xylene and the slides were coverslipped with Permount (Fisher Scientific).

STATISTICAL ANALYSIS

Statistical differences with respect to the focus forming assays were performed by Analysis of Variance followed by independent t-test. Differences between vehicle and rapamycin-treated groups in the transplanted tumor models were also perfomed by Analysis of Variance followed by independent t-test. The survival curve was analyzed using a log rank test. Variance estimates presented in all graphs were standard deviations.

CHAPTER 3

The PI-3K/mTOR pathway is involved in the regulation of ErbB3 during Neu-mediated mammary tumorigenesis

3.1 INTRODUCTION

A hallmark of the malignant phenotype is autonomous or unchecked cellular growth. Various components of the proliferative and survival pathways are constitutively activated or dysregulated in most human malignancies (Hanahan and Weinberg, 2000). Breast cancer provides one of the most well studied models which illustrate many levels of pathway dysregulation. These levels include cell membrane receptors, hormone receptors, cytoplasmic downstream circuitry, phosphatidylinositol 3-kinase and terminal elements that interact with nuclear targets (reviewed in (Atalay et al., 2003; Hidalgo and Rowinsky, 2000; Huang and Houghton, 2003; Mita et al., 2003a). Of relevance to this chapter is the observation that activating RTKs are amplified in the genesis of human breast cancer. In this regard, overexpression of wild-type ErbB2, generally attributable to gene amplification, occurs in 25-30% of human breast cancer and correlates with short time to relapse and lower overall survival (King et al., 1985; Slamon et al., 1987; Slamon et al., 1989; Yokota et al., 1986). The generation of transgenic mice expressing wild type Neu (the rat homologue of ErbB2) in the mammary gland confirm the oncogenic potential of the Neu receptor in this tissue. Furthermore, activating mutations within the extracellular domain of Neu were discovered in 65% of the mammary tumors arising in the MMTV/wt Neu strain (Siegel et al., 1994). Moreover, these altered Neu receptors had the capacity to induce tumors (Siegel et al., 1994) through the formation of constitutive homodimers when expressed in the mammary gland of transgenic mice (Siegel and Muller, 1996) suggesting that the activating mutations in Neu play a causal role in the transformation process. Characterization of these altered Neu receptors revealed a potential collaborating event during Neu-mediated tumorigenesis.

Strikingly, endogenous ErbB3 levels have been shown to be uniformly upregulated by 10-15 fold in mammary tumors overexpressing activated Neu when

compared to normal adjacent tissue (Siegel et al., 1999). The presence of equivalent levels of ErbB3 transcript in normal versus tumor tissues suggests that this increase is a result of either increase translation or stability of the ErbB3 protein. Furthermore, the overexpressed ErbB3 is constitutively tyrosine phosphorylated suggesting an active signaling role for this receptor in Neu-mediated transformation (Siegel et al., 1999). ErbB3 itself however, has impaired tyrosine kinase activity (Guy et al., 1994b) and needs a dimerization partner to become phosphorylated and acquire signaling potential (Kim et al., 1998). These observations compliment published results demonstrating elevated levels of ErbB2 and ErbB3 in sporadic human cancers (Naidu et al., 1998; Siegel et al., 1999).

The significance of Neu and ErbB3 co-expression is unclear. Therefore, the overall goal of this thesis is to address the molecular pathway of ErbB3 regulation and its role during Neu-mediated tumorigenesis. One potential explanation for Neu/ErbB3 co-expression may be that they recruit signaling pathways that cooperate during mammary tumorigenesis. Studies have shown that Neu induces transformation by sending strong proliferative signals predominantly through the Ras/MAPK pathway (Dankort et al., 1997). ErbB3, on the other hand, may be sending strong survival signals by recruiting the PI-3K-dependent antiapoptotic signaling pathway. Studies clearly demonstrate that ErbB3 has six consensus binding motifs that are specific for PI-3K and that ErbB3 is the preferred binding partner for PI-3K versus the other EGFR members (Fedi et al., 1994; Soltoff et al., 1994). Therefore, there is a strong possibility that ErbB3, in conjunction with Neu, activates the PI-3K-dependent cell survival circuitry and helps Neu in reaching its full oncogenic potential. Indeed, transgenic mouse models for breast cancer support the notion that both proliferative and survival signals must be activated within a cell before complete malignant transformation can occur (Amundadottir et al., 1996; Webster et al., 1998).

Cell proliferation and cell survival are distinct yet coupled processes that need to go hand in hand to induce transformation. There is accumulating evidence implicating that just as cdks are central regulators of cell proliferation, the mammalian target of rapamycin (mTOR) may be the central regulator of cell growth and survival. The PI-3K/Akt pathway has been implicated in the phosphorylation and activation of mTOR.

This subsequently leads to increased phosphorylation of 4EBP1 and S6K, both of which are important steps in the stimulation of protein translation (Gingras et al., 1998; Raught and Gingras, 1999). Since ErbB3 can bind and activate PI 3'K directly (Fedi et al., 1994; Kim et al., 1994; Soltoff et al., 1994), inactivation of 4E-BP1 and activation of S6K may, in part, account for elevated levels of ErbB3 protein in Neu-induced tumors. Furthermore, ErbB3 may be involved in activating mTOR targets that impinge on cell survival pathways.

Aberrant signaling of the PI-3K/mTOR signaling pathways often distinguish malignant from normal cells and therefore may represent possible strategic targets for therapeutic developments. Indeed, the mTOR inhibitor rapamycin, has demonstrated impressive antiproliferative actions in a diverse range of experimental tumors (Dilling et al., 1994; Eng et al., 1984; Guba et al., 2002; Mohi et al., 2004; Muthukkumar et al., 1995; Panwalkar et al., 2004; Seufferlein and Rozengurt, 1996; Wiederrecht et al., 1995; Yu et al., 2001a). Although the antitumor action of rapamycin has been attributed to its ability to modulate protein translation and G1 to S cell cycle phase traverse (Wiederrecht et al., 1995), the mechanism of how rapamycin inhibits tumor growth remains poorly understood. The rapamycin analog, CCI-779, is currently being evaluated in phase I/II clinical trials and although it shows some promise as a potential therapeutic strategy, a better understanding of the mechanism of mTOR regulation will enable targeting of those patients that will more favourably respond to CCI-779 treatment.

The results illustrated in this chapter suggest the following (1) Both PI-3K and mTOR are involved in the regulation of ErbB3 protein levels. (2) Inhibition of PI-3K leads to apoptotic cell death which implicates PI-3K in the cell survival circuitry in Neu-induced tumors. (3) Inhibition of mTOR by rapamcyin induces G1 arrest in a Neu-induced tumor cell line and inhibits the growth of transplanted tumors in vivo. (4) Importantly, rapamycin delays the onset and progression of tumors in a transgenic mouse model. (4) Interestingly, this decrease in tumor progression correlates with decreased expression of ErbB3 and cyclin D1. (5) The rapamycin-induced delay in tumor onset and progression in Neu-mediated transgenic mice also correlates with dephosphorylation of S6K at the rapamycin-sensitive Thr 389 only without affecting phosphorylation of

Thr421/424. (6) There is also a correlation between tumor inhibition and increased levels of hypophosphorylated 4EBP1 in transgenic mice treated with rapamycin.

In summary this chapter implicates the PI-3K/mTOR pathway in the regulation of ErbB3 protein. Furthermore, it suggests that downregulation of ErbB3 may represent one additional mechanism by which rapamycin works to inhibit ErbB2-mediated tumor growth.

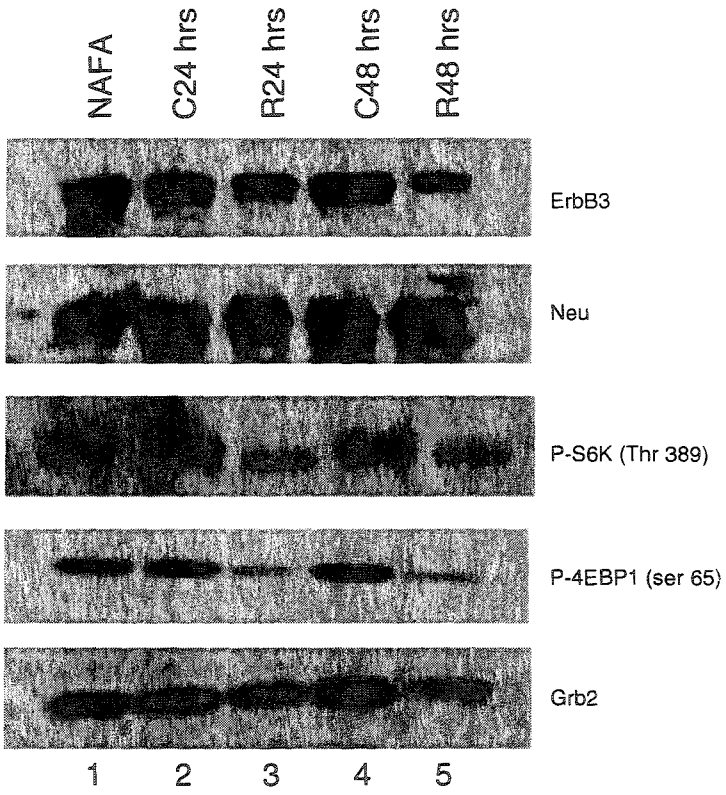
3.2 RESULTS

3.2.1 Rapamycin decreases levels of ErbB-3 protein and dephosphorylates S6K and 4EBP1.

As mentioned earlier, Neu-induced tumors show a 10-15 fold increase in endogenous ErbB3 protein levels highlighting the importance of better understanding the molecular mechanism of ErbB3 regulation in these tumors. Since ErbB3 contains six consensus binding motifs for PI-3K and since PI-3K activates mTOR, we first tested whether mTOR was involved in regulating and sustaining the high levels of ErbB3 protein observed in Neu-induced tumors. Cell lines derived from MMTVNeuNT mice (NAFA cells) were used to study ErbB3 regulation. The kinase activity of mTOR was inhibited by use of its specific inhibitor, rapamycin. NAFA cells treated with DMSO alone were used as controls for each timepoint. This would assure that any changes observed are due to rapamycin alone and not DMSO. As shown in Figure 3.1, NAFA cells treated with rapamcyin show a significant decrease in the levels of ErbB3 protein at 24 and 48 hrs. Having seen a change in ErbB3 protein levels after rapamycin treatment, we examined whether this effect was a general phenomenon for other receptor tyrosine kinases as well. Interestingly, levels of Neu remained unchanged upon rapamycin treatment of NAFA cells suggesting specificity of mTOR for ErbB3. This result supports the hypothesis that mTOR is involved in the regulation of ErbB-3 in these tumors.

As mentioned earlier, 4EBP1 and p70S6 kinase are the primary downstream effectors of mTOR and are involved in the regulation of protein translation and their phosphorylation statuses are often used as a read-out for mTOR kinase activity. We

Figure 3.1. Rapamycin decreases levels of ErbB-3 protein and dephosphorylates S6K and 4EBP1. Western immunoblot showing changes in ErbB3, Neu, phospho-S6K and phospho-4EBP1 after treatment of NAFA cells with rapamycin (40 ng/ml). Cells were lysed at 24 and 48 hrs. Equivalent amounts of protein (50 μg) from lysates were electrophoresed on a 4-12% gradient SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The membrane was probed with either ErbB-3, Neu, phospho-S6K (Thr389), phospho-4EBP1(Ser65) or Grb2-specific antibodies. Grb-2 immunoblot was used as protein loading control. NAFA (untreated cells), C24 hrs (treatment with DMSO for 24 hrs), R24 hrs (treatment with rapamycin for 24 hrs), C48 hrs (treatment with DMSO for 48 hrs), R48 hrs (treatment with rapamycin for 48 hrs).

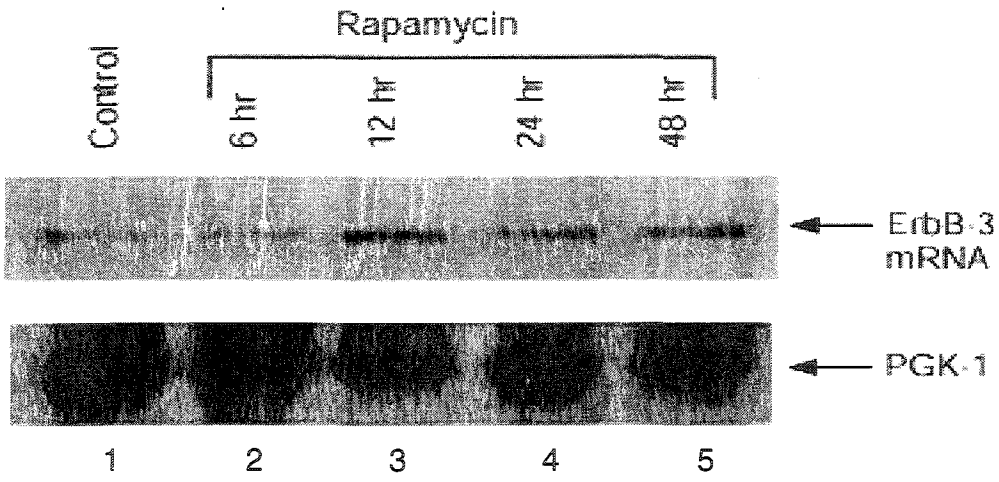

NAFA
C24 hrs
R24 hrs
C48 hrs
R48 hrs
ErbB3
Neu
P-S6K (Thr 389)
P-4EBP1 (ser 65)
Grb2
1
2
3
4
5

tested whether NAFA cells were sensitive to rapamycin treatment. Indeed there are many cell lines that are resistant to rapamycin treatment as observed by the inability of rapamycin to dephosphorylate rapamycin-sensitive Ser/Thr phosphorylation sites on these translational effectors. In order to test the sensitivity of NAFA cells to rapamycin, NAFA cells were treated with rapamycin and protein extracts were subjected to immunoblot analysis using 4EBP1 and S6K phospho-specific antibodies to sites Ser65 and Thr 389, respectively. These sites were chosen because both sites are indicative of kinase activity. As mentioned earlier, of the four phosphorylation events required to 4EBP1 inactivation, phosphorylation of Ser 65 allows it to dissociate from its target eIF4E and allows translation to occur. Similarly, Thr 389 phosphorylation most closely parallels kinase function of S6K. Figure 3.1 shows that inhibition of mTOR by rapamycin decreases the phosphorylation statuses of both 4EBP1 and S6K on Ser65 and Thr 389 sites at 24 and 48 hr time-points. This strongly shows that the NAFA cell line is rapamycin-sensitive and most importantly, dephosphorylation of 4EBP1 and S6K may, in part, play a role in the decrease in ErbB3 observed upon rapamycin treatment.

3.2.2 Rapamycin does not alter ErbB3 mRNA levels

As mentioned earlier, the dramatic increase in endogenous ErbB3 protein levels observed in Neu-induced tumors does not arise from an increase in its transcript levels. This strongly suggests a translational or post-translational regulation of ErbB3. Since we hypothesize that ErbB3 is regulated at the protein level by PI-3K/mTOR pathway, inhibition of mTOR should not lead to changes in ErbB3 transcript levels. In order to assess this, NAFA cells were treated with rapamycin at a concentration of 40 ng/ml and the cells were lysed at various time-points (6, 12, 24, 48 hrs) and total RNA was extracted and subjected to RNase protection analysis using radiolabeled ErbB3-specific riboprobe. The results of this analysis revealed that rapamycin does not affect ErbB-3 mRNA levels in NAFA cells (Figure 3.2). This strongly suggests that mTOR is not involved in regulating ErbB3 at the mRNA level but rather at the translational or post-translational level.

Figure 3.2. The levels of ErbB-3 transcript remain unchanged after treatment with rapamycin. NAFA cells were treated with rapamycin (40 ng/ml) and the cells were lysed at different time periods (6, 12, 24, 48 hrs). Total RNA was isolated from these cells and levels of ErbB-3 transcripts were determined by RNase protection analysis. Total RNA (15 μg) was hybridized to an antisense ErbB-3 riboprobe. The protected fragment corresponding to the ErbB-3 message is indicated. An antisense internal control probe directed against the mouse phosphoglycerate kinase (PGK) gene was included to control for equal loading of RNA onto the gel.

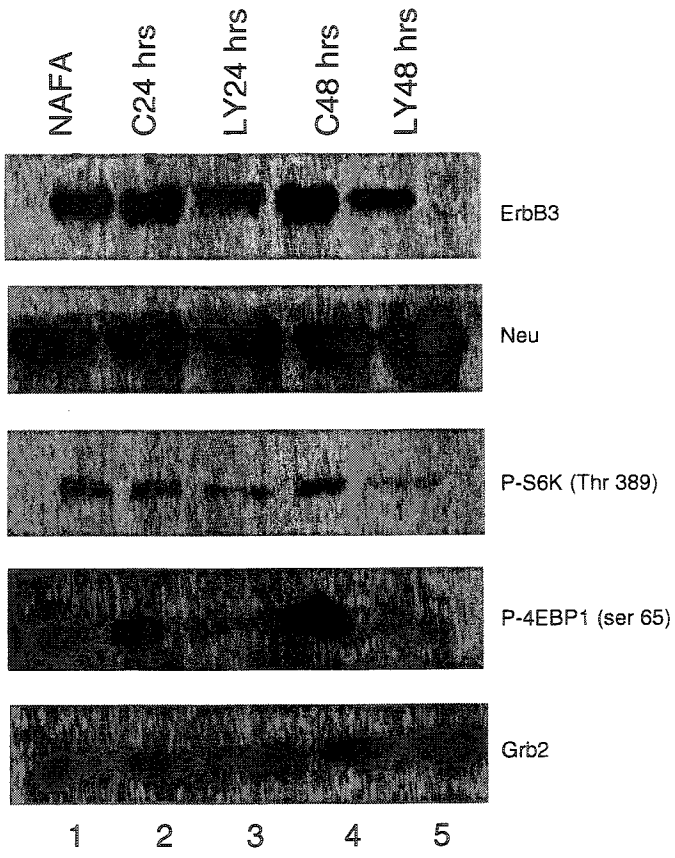

Rapamycin
Control
6 hr
12 hr
24 hr
48 hr
ErbB-3 mRNA
PGK-1
1
2
3
4
5

3.2.3 Inhibition of PI-3K by LY294002 decreases ErbB3 protein levels and dephosphorylates S6K and 4EBP1.

While our observations so far suggest that ErbB3 protein levels are regulated by the mTOR/4EBP1 pathway, there is no direct evidence to suggest that it is funneling through PI-3K. Since ErbB3 is a likely binding partner of PI-3K, it is very probable that PI-3K may play a role in modulation of ErbB3 protein levels. However, we cannot rule out the fact that other pathways may be predominantly responsible. Indeed studies have shown that members of the Ras/MAPK pathway also activate the mTOR/4EBP1 pathway directly. Since this pathway is upregulated in Neu-induced tumors, there is a possibility that it may play a significant role. Therefore, in order to directly assess whether PI-3K is involved in the regulation of ErbB3, we inhibited PI-3K by its specific inhibitor LY294002 in NAFA cells and determined the levels of ErbB3 and Neu proteins. The results shown in Figure 3.3 demonstrate that ErbB-3 protein levels decrease after treatment with LY294002. Similar to rapamycin treatment, LY294002 also does not have an effect on the levels of Neu protein which strongly suggests that PI-3K specifically regulates the ErbB3 receptor tyrosine kinase.

As mentioned above, treatment of NAFA cells with LY294002 led to a significant decrease in the levels of ErbB3 protein suggesting a role for PI-3K in ErbB3 protein regulation. Several reports strongly suggest that PI-3K is directly involved in the phosphorylation of S6K. In order to assess whether the decrease in ErbB3 observed upon LY294002 treatment is due to dephosphorylation of 4EBP1 and S6K, NAFA cells were treated with LY294002 and the protein extracts were subjected to immunoblot analysis using phospho-specific antibodies for 4EBP1 and S6K. Figure 3.3 shows that LY294002 leads to dephosphorylation of 4EBP1 at the Ser65 site which suggests that it is activated and bound to eIF4E and may perhaps play a role in suppression of ErbB3 translation. Similarly, dephosphorylation of the kinase function indicative site Thr 389 on S6K suggests that it too may play a role in ErbB3 protein regulation. These results most certainly argue that PI-3K is involved in regulating both the downstream translational effectors of mTOR in NAFA cells. In particular, it inactivates 4EBP1 and activates S6K, both of which are required for translation of proteins involved

Figure 3.3. LY294002 decreases ErbB3 protein levels and leads to dephosphorylation of S6K and 4EBP1. Western immunoblot showing changes in ErbB3, Neu, phospho-S6K and phospho-4EBP1 after treatment of NAFA cells with LY294002 (50μM). Cells were lysed at 24 and 48 hrs. Equivalent amounts of protein (50 μg) from lysates were electrophoresed on a 4-12% gradient SDDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The membrane was probed with either ErbB-3, Neu, phospho-S6K (Thr389), phospho-4EBP1(Ser65) or Grb2-specific antibodies. Grb-2 immunoblot was used as protein loading control. NAFA (untreated cells), C24 hrs (treatment with DMSO for 24 hrs), LY24 hrs (treatment with LY294002 for 24 hrs), C48 hrs (treatment with DMSO for 48 hrs), LY48 hrs (treatment with LY294002 for 48 hrs).


NAFA
C24 hrs
LY24 hrs
C48 hrs
LY48 hrs
ErbB3
Neu
P-S6K (Thr 389)
P-4EBP1 (ser 65)
Grb2
1
2
3
4
5

in cell growth and survival. This suggests that the PI-3K itself is involved in the regulation of ErbB3 protein levels and may in part, play a role in sustaining the high levels of ErbB3 protein observed during Neu-mediated mammary tumorigenesis.

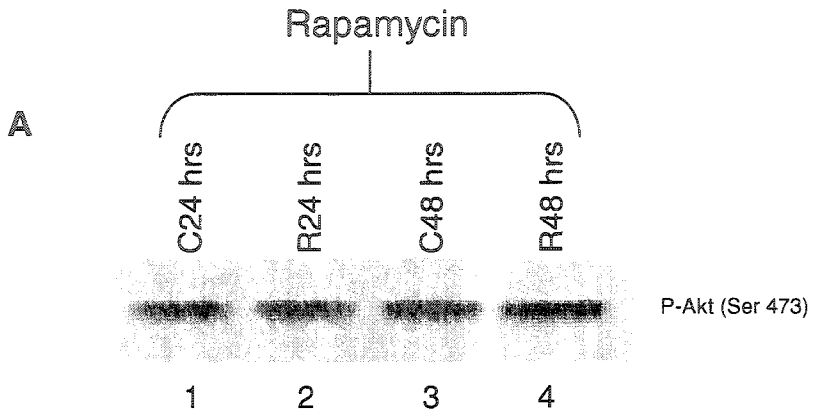
3.2.4 Rapamycin and LY294002 do not affect Akt phosphorylation

Figure 3.4A suggests that inhibition of mTOR does not affect the phosphorylation state of Akt. The hypothetical model suggests that Akt is the mediator between ErbB3/PI-3K and mTOR signaling. Since Akt is upstream of mTOR, it is very much possible that rapamycin would not have a significant effect on Akt. According to the hypothetical model, Akt-mediated regulation of mTOR phosphorylation is dependent upon activation of PI-3K. Therefore, inhibition of PI-3K using LY294002 should have a significant effect on Akt phosphorylation. However, this is not the case. Figure 3.4B clearly demonstrates that Akt is not dephosphorylated upon PI-3K inhibition in NAFA cells suggesting an alternative pathway connecting PI-3K to mTOR.

3.2.5 Dominant negative PI-3K inhibits ErbB-3 protein in NAFA cells

In order to confirm that the decrease in ErbB-3 observed is due to specific inhibition of PI-3K, stable cell lines expressing the inducible dominant negative PI-3K (Δp85) were derived using NAFA cells. Since PI-3K is involved in the anti-apoptotic/cell survival pathway, expression of this mutant would not be compatible with cell survival, and hence an inducible form was constructed as shown in Figure 3.5A. This mutant contains mutations in the p85 regulatory unit which prevents it from binding to the p110 catalytic subunit. The inducible nature of Δp85 derives from the presence of loxP sites which are natural targets of Cre recombinase. Therefore, infection of Δp85 NAFA cells with AdCre leads to excision of the Neo cassette and subsequent transcription of Δp85 under the control of MoMULV-LTR promoter. NAFA cell lines that inducibly express the Δp85 mutant, were derived as described in section 2.3.1. Complete excision of the neomycin cassette occured within 48 hrs. To determine whether the dominant negative

Figure 3.4. Rapamycin and LY294002 do not affect Akt phosphorylation. Western immunoblot showing changes in Akt phosphorylation after treatment of NAFA cells with rapamycin (40 ng/ml)(R) (A) or LY294002 (50μM) (LY) (B). Cells were lysed at 24 and 48 hrs. Equivalent amounts of protein (50 μg) from lysates were electrophoresed on a 4-12% gradient SDDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The membrane was probed with phospho-Akt (Ser473)-specific antibodies. C24 hrs (treatment with DMSO for 24 hrs), R24 or LY24 hrs (treatment with rapamycin or LY294002 for 24 hrs), C48 hrs (treatment with DMSO for 48 hrs), R48 or LY24 hrs (treatment with rapamycin or LY294002 for 48 hrs).


A
Rapamycin
C24 hrs
R24 hrs
C48 hrs
R48 hrs
P-Akt (Ser 473)
1
2
3
4

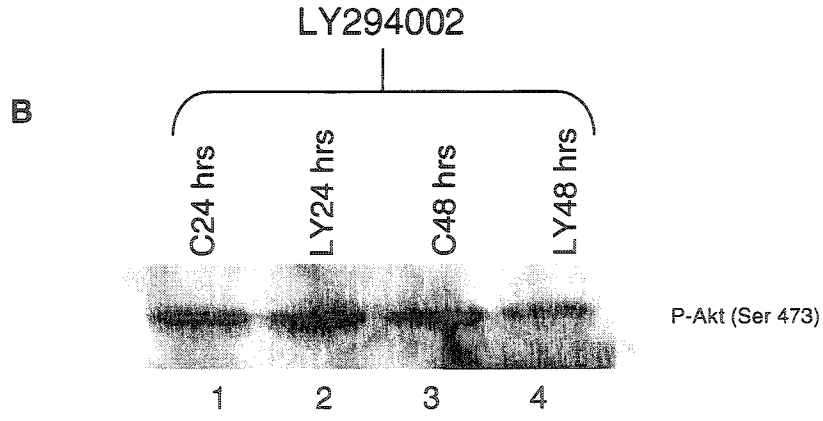
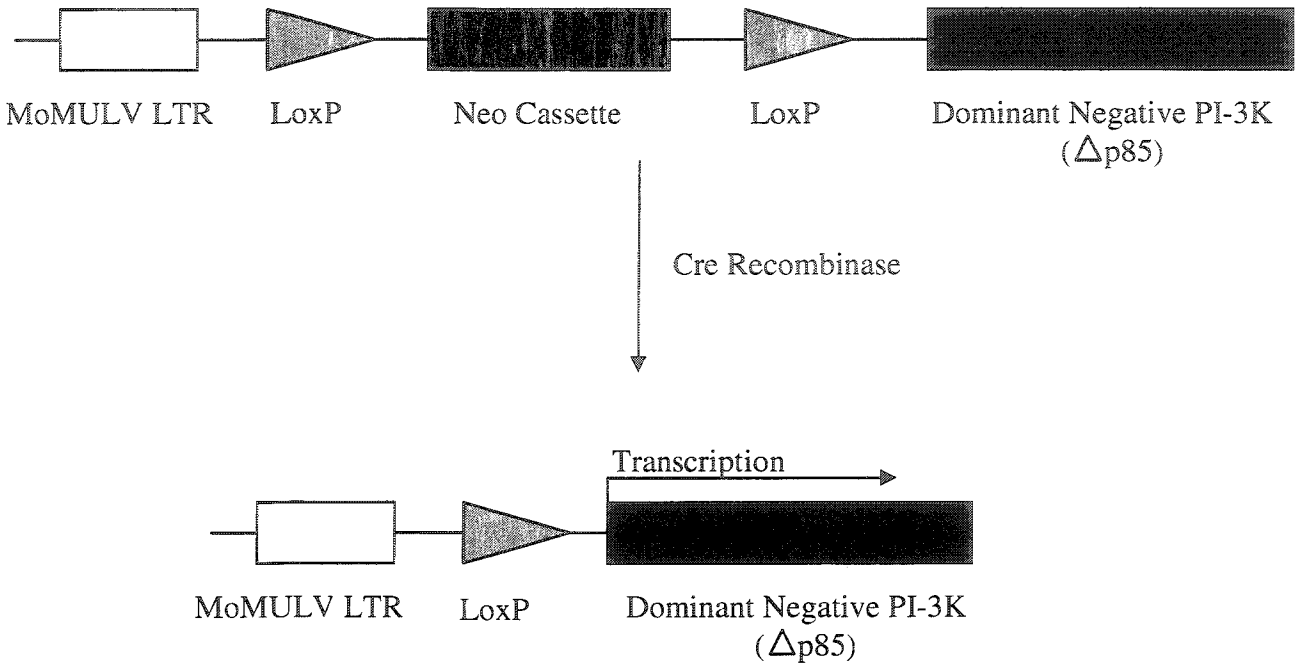
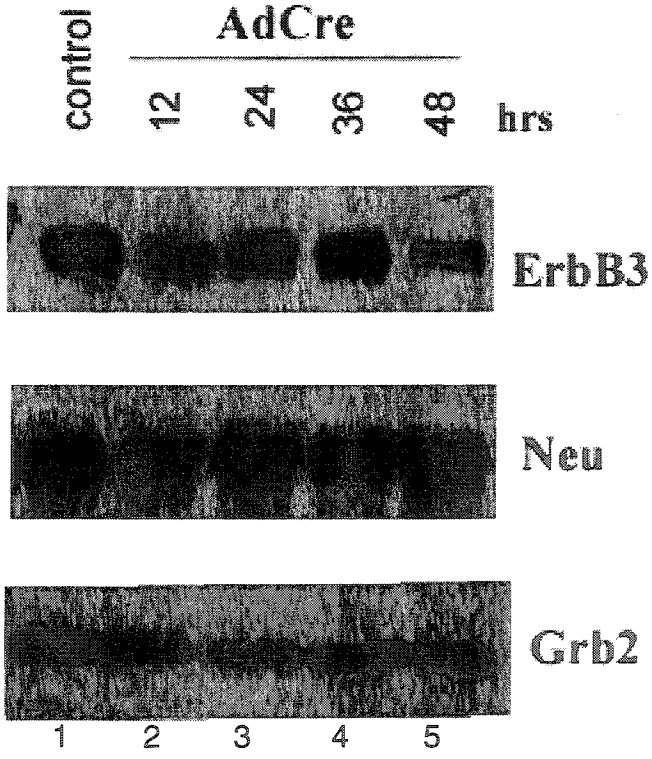

B
LY294002
C24 hrs
LY24 hrs
C48 hrs
LY48 hrs
P-Akt (Ser 473)
1
2
3
4

Figure 3.5. Schematic illustration of Cre-inducible expression cassette carrying the dominant negative PI-3K and its effects on levels of ErbB3 protein. (A) Stable cell lines expressing the inducible dominant –ve PI-3K (Δp85) were derived using NAFA cells. This mutant contains mutations in the Src homology 2 bearing p85 regulatory unit which prevents association with the p110 catalytic subunit. The inducible nature of Δp85 derives from the presence of loxP sites which are natural targets of Cre recombinase. Therefore, infection of Δp85 NAFA cells with AdCre leads to excision of the Neo cassette and subsequent transcription of Δp85 under the control of MoMULV-LTR promoter. **(B)**Western immunoblot showing that infection of NAFA cells with AdCre (moi=20) for 48 hrs leads to a decrease in ErbB3 but not Neu protein levels. Equivalent amounts of protein (50 μg) from lysates were electrophoresed on a 4-12% gradient SDDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The blot was probed with either ErbB3, Neu or Grb2-specific antibodies. Grb-2 immunoblot was used to confirm that equivalent amounts of protein were present in each lane.

A


MoMULV LTR
LoxP
Neo Cassette
LoxP
Dominant Negative PI-3K
(Δp85)
Cre Recombinase
Transcription
MoMULV LTR
LoxP
Dominant Negative PI-3K
(Δp85)

B


control
AdCre
12
24
36
48
hrs
ErbB3
Neu
Grb2
1
2
3
4
5

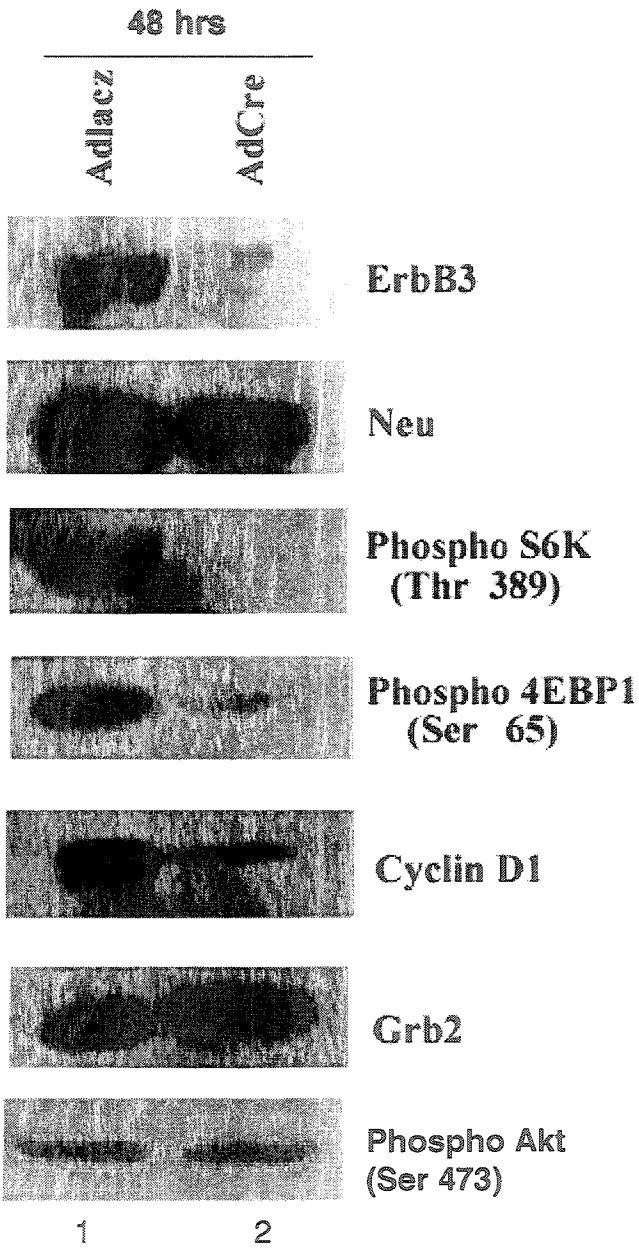
PI-3K leads to a decrease in ErbB3 protein levels, Δp85NAFA cells were infected with AdCre and the cells were lysed at various time-points (12, 24, 36, 48 hrs). The results in Figure 3.5B demonstrate that AdCre expression leads to a specific decrease in levels of ErbB3 protein. There are no changes in ErbB3 levels at 12, 24 and 36 hrs suggesting that the levels of ErbB3 only start to decrease anywhere between 36-48 hrs and by the 48 hr time-point there is a clear decrease in ErbB3 protein.

After determining that ErbB3 levels decrease after full excision by the AdCre virus, the 48 hr timepoint was used in the second set of experiments to confirm these results using proper adenovirus control. An adenovirus expressing β-galactosidase reporter (Adlacz) was used as a control to ensure that any changes observed were due to Cre expression and subsequent excision and expression of the mutant PI-3K. Figure 3.6 confirms that expression of the dominant negative PI-3K leads to a subsequent decrease in ErbB3 levels after 48 hrs. This effect is not due to presence of any adenovirus since Adlacz had no effect on ErbB3 levels. Interestingly, we observe similar results with the Δp85 mutant as we do with LY294002 in that we see no changes in the levels of Neu suggesting specificity of PI-3K-induced regulation for ErbB-3. These results strongly support the notion that PI-3K is involved in ErbB3 protein regulation in Neu-induced tumors.

3.2.6 Dominant negative PI-3K affects S6K, 4EBP1 and cyclin D1 but not Akt

Previous studies have shown that PI-3K is involved in the regulation of the two important mTOR translational effectors, S6K and 4EBP1. PI-3K is thought to be directly involved in the phosphorylation of Thr389 site on S6K and indirectly phosphorylates 4EBP1 by activating mTOR. Since the Δp85 mutant led to a decrease in ErbB3 protein levels, we examined whether this decrease may, in part, be due to dephosphorylation of S6K and 4EBP1. The results in Figure 3.6 show that expression of the PI-3K mutant leads to complete dephosphorylation both S6K and 4EBP1. Since phosphorylation of Thr 389 and Ser 65 closely parallel the function of these protein, these results suggest that PI-3K may be involved in the regulation of S6K and 4EBP1 activity in Neu-mediated transformation.

Figure 3.6. Biochemical analysis of NAFA cells possessing the inducible dominant negative p85 inhibitor. Western immunoblot showing effects of Adcre (48 hrs) on ErbB3, Neu, Akt and components of the PI3'K/mTOR pathway. Equivalent amounts of protein lysates (50μg) from AdCre-infected Δp85NAFA cells were electrophoresed on a 4-12% gradient SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The membrane was probed with either ErbB-3, Neu, Akt, phosphoS6K (Thr389), phospho4EBP1(Ser65) or cyclin D1-specific antibodies. Grb-2 immunoblot was used to confirm that equivalent amounts of protein were present in each lane.


48 hrs
Adlacz
AdCre
ErbB3
Neu
Phospho S6K
(Thr 389)
Phospho 4EBP1
(Ser 65)
Cyclin D1
Grb2
Phospho Akt
(Ser 473)
1
2

Several studies have shown that activation of PI-3K leads to phosphorylation of Akt. To confirm that Akt was involved in linking PI-3K to the mTOR/4EBP1 pathway, the phosphorylation status of Akt at Ser 473 was determined in NAFA cells expressing an inducible dominant negative PI-3K (Δp85). Figure 3.6 illustrates that Δp85 had no affect on Akt phosphorylation suggesting that PI-3K impinges on S6K and 4EBP1 by alternate mechanisms that are independent of Akt.

Taken together, the results of figure 3.6 suggest that the role of PI-3K in these Neu-induced tumors may be to sustain high levels of ErbB3 protein by upregulating mTOR and its downstream effectors, S6K and 4EBP1.

3.2.7 Both rapamycin and LY294002 increase ErbB-3 turnover.

So far the results suggest that PI3K and mTOR are involved in the regulation of ErbB3 protein. However, whether this regulation is at the translational and/or post-translational level remains to be addressed. Indeed, studies have shown that mTOR regulates the translation as well as post-translation of specific proteins. In order to determine whether the rapamycin and LY294002-induced decrease in ErbB-3 protein levels in NAFA cells was due to an increase in protein turnover, NAFA cells were first treated with either DMSO alone, rapamycin or LY294002 and pulse-labeled with ^{35}S methionine. There is a significant decrease in ^{35}S-labeled ErbB3 at 12 hours when compared to the control (NAFA) (Figure 3.7). As time goes by, there is a further decrease in ^{35}S-labeled ErbB3 suggesting an increase in its turnover over the course of 48 hrs. ErbB3 turnover started to increase anywhere between 6-12 hrs when compared to the control (NAFA) and continued to increase at 24 and 48 hrs post rapamycin treatment. Treatment of NAFA cells with LY294002 also led to a decrease in ^{35}S-labeled ErbB3 at the 48 hr timepoint compared to the NAFA control suggesting an increase in its turnover. Both rapamycin and LY294002 had comparable effects on ErbB3 turnover. The results of this pulse-chase analysis study suggest that both PI-3K and mTOR are involved in the regulation of ErbB3 at the post-translational level. Therefore, there is a possibility that the dramatic increase in endogenous ErbB3 proteins observed in Neu-induced tumors may, in part, be

due to a decrease in its turnover. Furthermore, both PI-3K and mTOR may play a role in sustaining high levels of ErbB3 by increasing the stability of this oncogene.

3.2.8 PI-3K inhibition induces apoptosis in NAFA cells

So far the biochemical data suggests that ErbB3 is regulated by the PI-3K/mTOR pathway. However, the biological significance of this regulation remains unknown. Since the hypothesis is that ErbB3 may be providing cell survival signals by coupling to the PI-3K/mTOR anti-apoptotic pathway, we determined whether inhibition of several different members of this pathway would result in apoptosis. The members that were targeted were Neu, PI-3K and mTOR. NAFA cells were treated with either rapamycin or LY294002 and cells were examined after 48 hrs for cell death due to apoptosis by performing a TUNEL assay. Furthermore, the inducible Δp85 dominant negative PI-3K mutant expressing NAFA cell line was infected with AdCre and apoptotic cell death was determined after 48 hrs. The results of this study are illustrated in Figure 3.8. The control panels were either treated with DMSO alone (NAFA) or Adlacz. The results clearly demonstrate that inhibition of PI-3K using LY294002 and the dominant negative mutant (Δp85) leads to apoptotic cell death. Rapamycin, however, did not induce apoptosis in NAFA cells. Taken together these results suggest that PI-3K signaling may be providing the necessary anti-apoptotic signals during Neu-mediated transformation.

3.2.9 Rapamcyin induces G1 arrest and inhibits cyclin D1 in NAFA cells

Results of the TUNNEL assay revealed that rapamycin does not lead to apoptotic cell death in NAFA cells. Therefore, the biological significance of mTOR inhibition in NAFA cells needed to be elucidated. Previous studies have shown that rapamycin leads to G1 arrest in certain cell types by predominantly impinging on the activity of cyclin D1. Therefore, we hypothesized that G1 arrest may be a biological outcome of mTOR inhibition in NAFA cells. Figure 3.9 illustrates the results of the FACS analysis. Figure 3.9A demonstrates the normal cell cycle distribution of NAFA cells treated with DMSO

Figure 3.7. Pulse chase analysis on NAFA cells showing that inhibition of PI-3K and mTOR leads to an increase in ErbB-3 turnover. The half-life of ErbB3 after treatment of NAFA cells with rapamycin and LY294002 was determined using pulse chase analysis. ^{35}S-labeled ErbB3 was transferred onto immobilon P membrane and transferred onto film. The amount of ^{35}S-labeled ErbB3 was quantified using a phosphoimager and summarized in the graph shown. Note the decrease in the ^{35}S-labeled ErbB3 upon treatment with rapamycin which starts between 6 and 12 hours. By 48 hrs, there is approximately a 90% decrease in ^{35}S-labeled ErbB3. Note the same for LY294002 when compared to untreated NAFA cells.

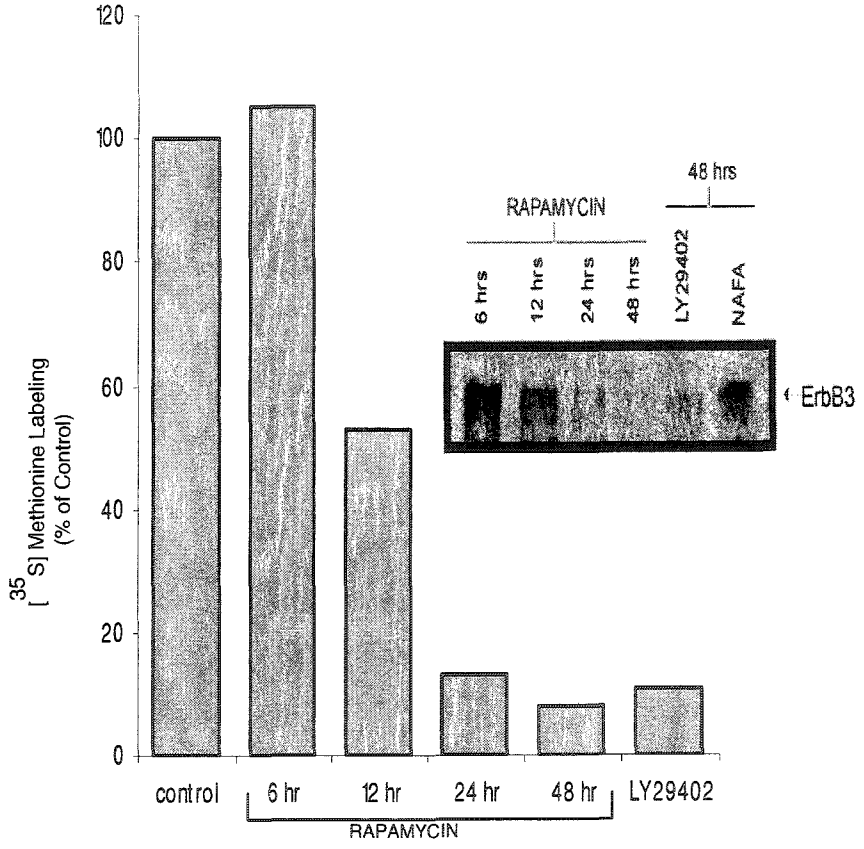

120
100
80
60
40
20
0
[35S] Methionine Labeling
(% of Control)
control
6 hr
12 hr
24 hr
48 hr
LY29402
RAPAMYCIN
48 hrs
RAPAMYCIN
6 hrs
12 hrs
24 hrs
48 hrs
LY29402
NAFA
ErbB3

Figure 3.8. Inhibition of PI-3K activity leads to apoptosis. TUNEL Assay was performed on NAFA cells treated with either DMSO alone (NAFA control), rapamycin or LY294002. NAFAΔp85 stable cell lines were infected with either control Adlacz virus or AdCre followed by TUNEL assay after 72 hrs. Both LY294002 and dominant negative PI-3K (Δp85) lead to apoptotic cell death as shown. Cells undergoing apoptosis have their nucleus stained dark brown. Note that rapamycin does not induce apoptosis in NAFA cells.

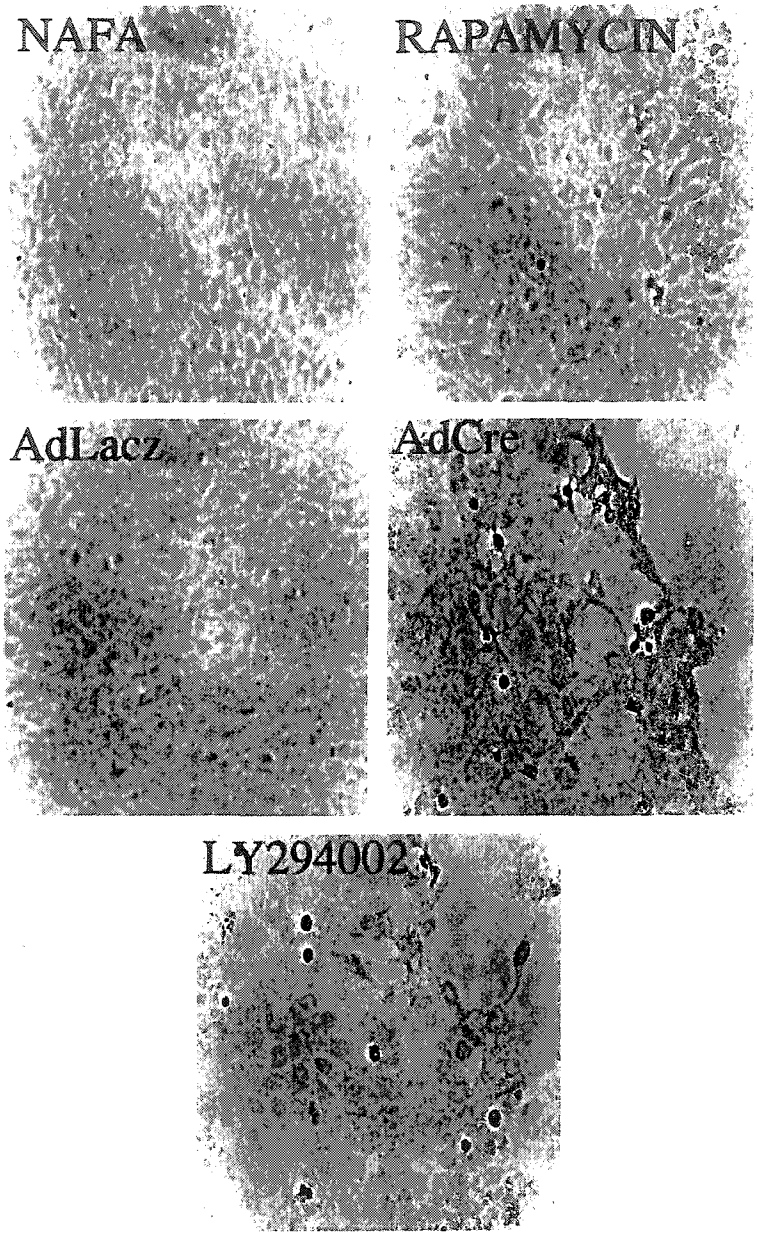
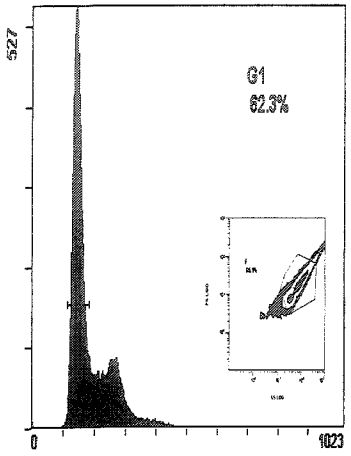
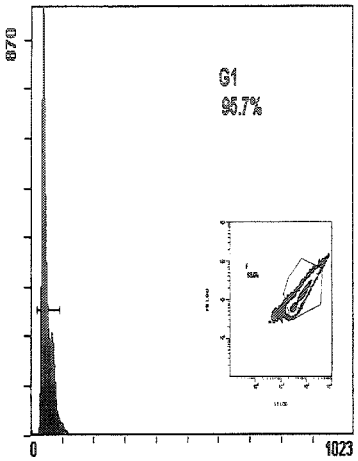

NAFA
RAPAMYCIN
AdLacz
AdCre
LY294002

Figure 3.9. Rapamycin induces G1 arrest in NAFA cells in a dose dependent manner. NAFA cells were treated with various concentrations of rapamycin for 24 hrs after which the cells were fixed by adding ice-cold ethanol and the DNA content of each sample (approximately 2×10^6 cells) was estimated by staining with propidium iodide and quantifying the fluorescence by the use of a Cell Quest FACScan flow cytometer. (A) FACS analysis showing normal cell cycle distribution of untreated NAFA cells. (B) Rapamycin (4μg/ml) arrests 96% of NAFA cells in G1 phase of the cell cycle. (C) Rapamycin leads to G1 arrest in a dose dependent manner.

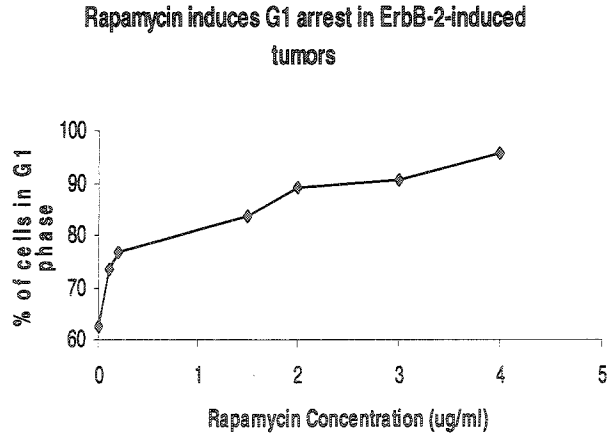
A


527
G1
62.3%
0
1023

B


870
G1
95.7%
0
1023

C


Rapamycin induces G1 arrest in ErbB-2-induced tumors
% of cells in G1 phase
100
90
80
70
60
0
1
2
3
4
5
Rapamycin Concentration (ug/ml)

only. The graph shows an expected distribution of cells in the G1, G2, S and M phases of the cell cycle. The phase of interest in this case is the G1 phase. The distribution shows that NAFA plates that have not been treated with rapamycin have approximately 63% of their total cells in G1 phase of the cell cycle. Treatment of NAFA cells with rapamycin (4μg/ml) leads to approximately 96% of cells in G1 phase (Figure 3.9B). This strongly suggests that inhibition of mTOR in NAFA cells leads to G1 cell cycle arrest. Furthermore, the percentage of cells in G1 phase depends on the concentration of rapamycin. Figure 3.9C shows a concentration dependent increase in G1 arrest upon rapamycin treatment.

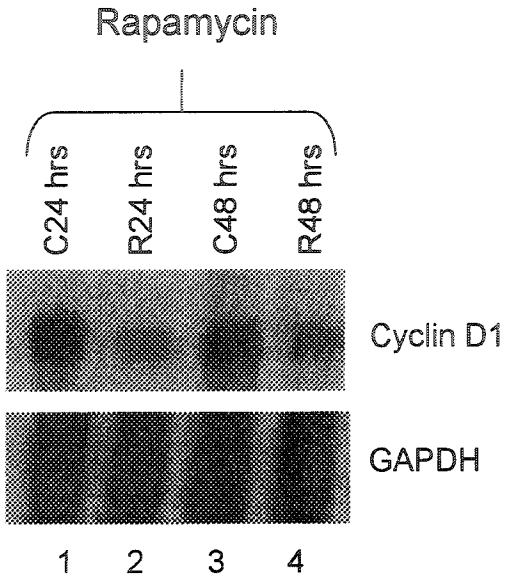
Studies have suggested that mTOR regulates cell cycle by exerting its effects on cyclin D1. Therefore, we determined whether rapamycin-induced inhibition of mTOR and the subsequent G1 arrest of NAFA cells was a result of cyclin D1 downregulation. The results revealed a decrease in cyclin D1 mRNA levels upon rapamycin treatment of NAFA cells. Figure 3.10A shows that control NAFA cells treated with DMSO only do not show changes in cyclin D1 mRNA even after 48 hours. However, at 24 hours there is a clear decrease in cyclin D1 mRNA. These changes were not a result of unequal RNA loading since GAPDH levels remain unchanged. We also determined whether mTOR regulated cyclin D1 at the protein level as well in NAFA cells. Protein extracts derived from rapamcyin treated NAFA cells were subjected to immunoblot analysis using cyclin D1-specific antibodies. The results of the western blot shown in Figure 3.10B demonstrate that rapamcyin substantially decreases levels of cyclin D1 protein. Control samples treated with DMSO alone remain unchanged. Cyclin D1 protein seems to almost be almost completely downregulated by 24 hours. Grb2 protein was used as a control of equal protein loading.

3.2.10 Rapamycin prevents tumor growth in vivo: Mouse rapamycin trial

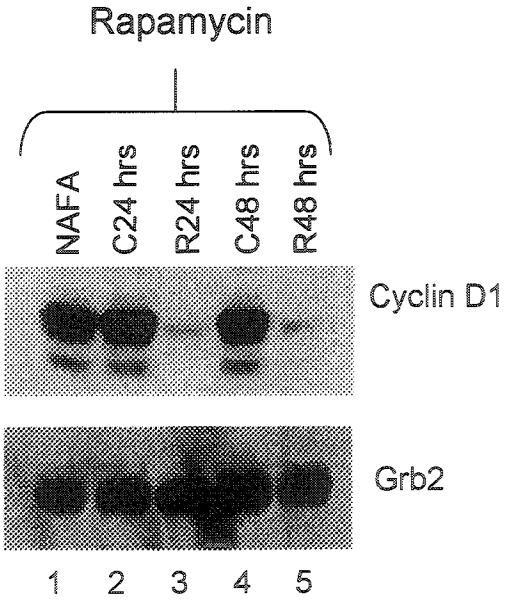
The above biochemical and biological data from rapamycin-treated NAFA cells led us to investigate whether rapamycin can inhibit NAFA cell tumor growth in a mouse randomized placebo controlled trial. Indeed studies have used rapamycin to inhibit tumor growth, however, it has been difficult to identify which tumors may be sensitive to

Figure 3.10. Rapamycin-treated NAFA cells show decreases in cyclin D1 mRNA and protein. (A) RNase protection showing that rapamycin decreases cyclin D1 mRNA. NAFA cells were treated with rapamycin. Total RNA was isolated from these cells and levels of cyclin D1 transcripts were determined by RNase protection analysis. Total RNA (15 μg) was hybridized to an antisense cyclin riboprobe. The protected fragment corresponding to the cyclin D1 message is indicated. An antisense internal control probe directed against the mouse GAPDH gene was included to control for equal loading of RNA onto the gel. (B) Western immunoblot showing that rapamycin decreases cyclin D1 protein. NAFA cells were treated with rapamycin. Equivalent amounts of protein (50 μg) from lysates were electrophoresed on a 4-12% gradient SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The blot was probed with cyclin D1-specific antibodies. Grb-2 immunoblots were used to confirm that equivalent amounts of protein were present in each lane.

A


Rapamycin
C24 hrs
R24 hrs
C48 hrs
R48 hrs
Cyclin D1
GAPDH
1
2
3
4

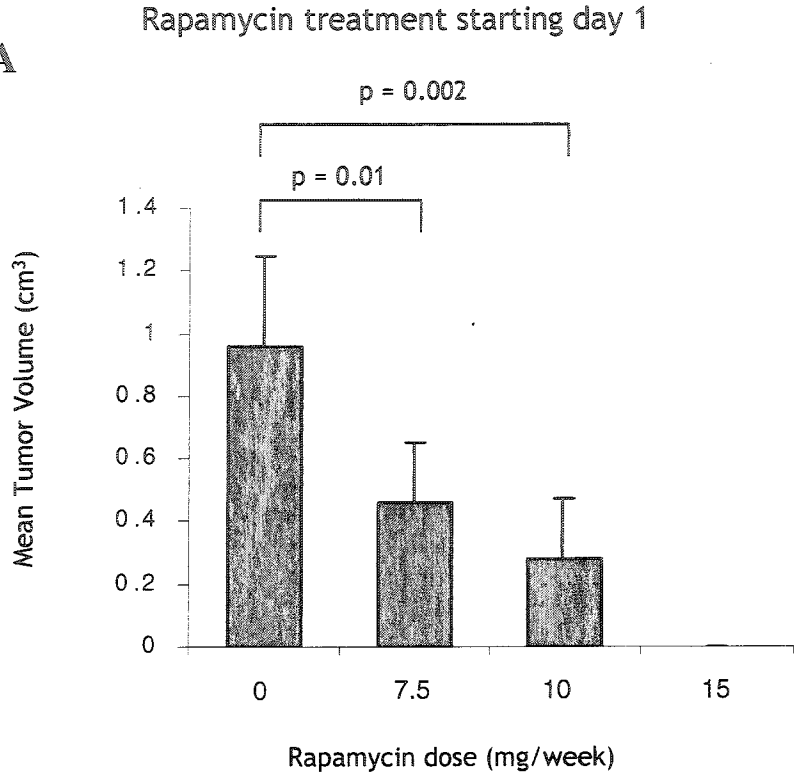
B


Rapamycin
NAFA
C24 hrs
R24 hrs
C48 hrs
R48 hrs
Cyclin D1
Grb2
1
2
3
4
5

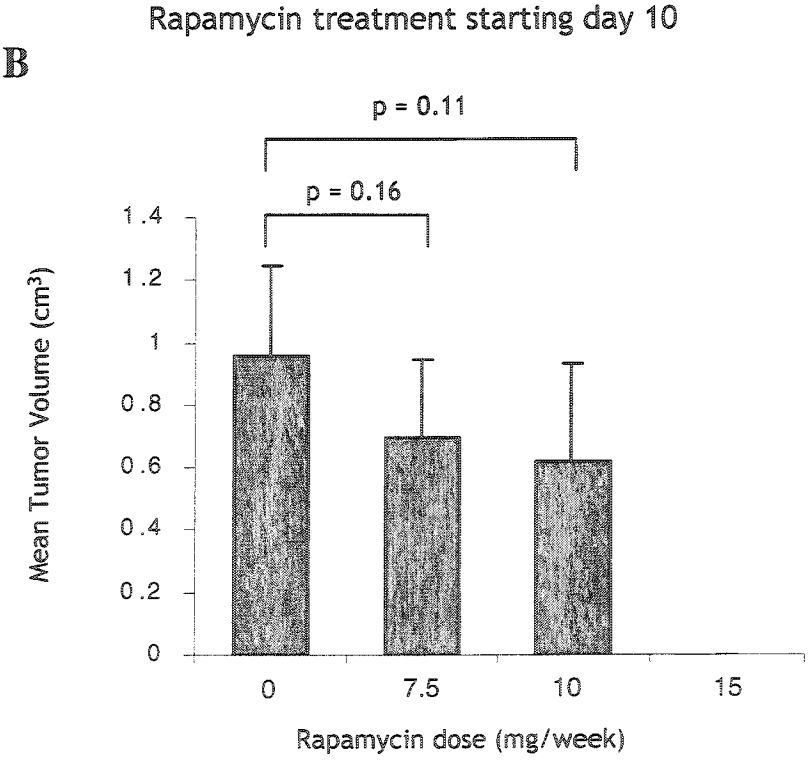
rapamycin and which tumors will most favourably respond to its antitumor effects. In order to do so, it is important to better understand the mechanism by which rapamycin may be inhibiting tumor growth. The above biochemical data suggests that one possible mechanism of rapamycin-induced tumor inhibition in ErbB2-mediated mammary tumors may involve downregulation of ErbB3 and thereby inhibiting the cell survival pathway. The goal in the subsequent experiments is to determine whether rapamycin can regress or inhibit tumor growth of a Neu-induced breast cancer cell line (NAFA cells) *in vivo*. To examine the antitumor effect of rapamycin *in vivo*, randomized placebo controlled trials were conducted on mice subcutaneously transplanted with NAFA cells. Two, four-arm parallel group was randomized to treatment with either placebo or 7.5, 10, 15 mg/kg/week of intraperitoneal rapamycin (Figure 3.11). Treatment with rapamycin began one day after NAFA cell transplantation in the first trial (Figure 3.11A), and ten days after transplantation in the second trial (Figure 3.11B). Results of the first arm of the study allow us to determine whether rapamycin inhibits the initiation of tumor growth. In the second arm, the tumors were allowed to grow for 10 days before rapamycin treatment began in order to determine whether rapamycin is capable of tumor regression. In both trials subcutaneous tumor volume was assessed with calipers three weeks after tumor transplantation. Figure 11A shows the results of the first trial. After 21 days, untreated mice show an average tumor growth of approximately $1cm^3$. Mice treated with 2.5 mg/kg/3xwk (total of 7.5 mg/kg/wk) of rapamycin show approximately 50% reduction in tumor size. Increasing the dose to 5 mg/kg/2xwk (total of 10 mg/kg/wk) leads to tumor inhibition by approximately 70% compared to the untreated group. The highest dose used was 5mg/kg/3xwk (total of 15mg/kg/wk). No tumor growth was detected at the end of week 2, however the mice were unable to reach study endpoint since they died from toxicity. Since therapy is a fine balance between effectiveness and toxicity, this study suggests that the most effective dose was 10mg/kg/wk. The results of the second arm of the trial in which rapamycin treatment started 10 days after tumor transplantation, are shown in Figure 11B. Similar to the previous group, untreated mice show a tumor growth of approximately 1 cm^3 at the end of 21 days. The group treated with 7.5 mg/kg/wk show about 25% reduction in tumor size. Increasing the dose to 10 mg/kg/wk did not substantially decrease the tumor size as it still remained approximately 25% reduced

Figure 3.11. Rapamycin prevents tumor growth *in vivo*. Bar graphs illustrating the effect of rapamycin on mice subcutaneously transplanted with NAFA cells. Four groups of five Fvb/n mice were transplanted with 10^6 NAFA cells subcutaneously in the right flank, and each group was randomized to treatment with vehicle, 7.5, 10 or 15 mg /kg/ week of intraperitoneal rapamycin. Treatment with rapamycin began one day after NAFA cell transplantation in the first trial (**A**), and ten days after transplantation in the second trial (**B**). Tumor size was measured on day 24 of study. Mice treated with 15 mg / week of intraperitoneal rapamycin demonstrated no detectable tumor, however did demonstrate signs of toxicity.

A


Rapamycin treatment starting day 1
p = 0.002
p = 0.01
Mean Tumor Volume (cm³)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
7.5
10
15
Rapamycin dose (mg/week)

B

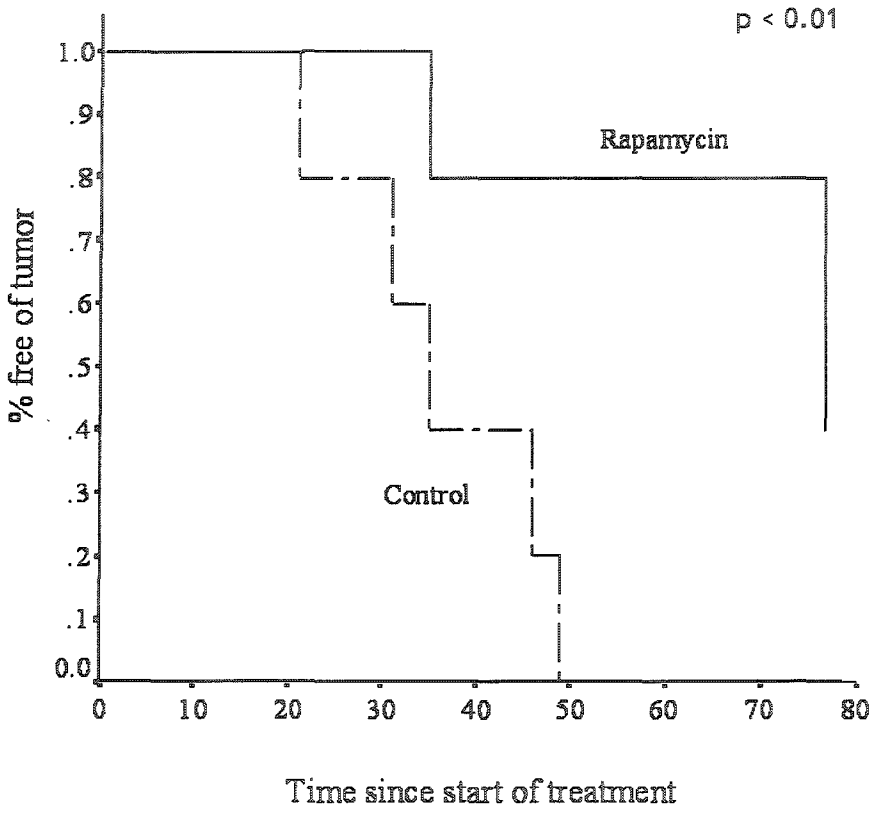

Rapamycin treatment starting day 10
p = 0.11
p = 0.16
Mean Tumor Volume (cm³)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
7.5
10
15
Rapamycin dose (mg/week)

compared to the untreated group. The results of these studies clearly indicate that rapamycin prevents *in vivo* tumor growth. The above results specifically show that rapamycin has a better effect on prevention of tumor growth than it does on tumor regression.

3.2.11 Rapamycin delays tumor onset and growth in transgenic mice overexpressing constitutively activated Neu in the mammary gland.

Given the promising results of the above transplanted mouse clinical trial, combined with the finding that rapamycin inhibits ErbB3, we attempted to determined whether rapamycin can delay or prevent the onset and progression of Neu-induced tumors originating from spontaneous somatic mutations in a transgenic mouse model. We next determined whether rapamycin-induced tumor inhibition in these transgenics was due to the mechanisms observed in tissue culture. The transgenic mouse model used was MMTV-NDL2-5 line of mice. These mice overexpress the activated Neu DeLetion mutant (NDL2-5) in their mammary glands. Previous studies have shown that the NDL2-5 transgenics develop tumors at an average of 161 (± 10) days (Siegel *et al*, 1996). Since the results of the NAFA isograft studies showed that rapamycin was better at preventing tumor growth than it was as regressing existing tumors, rapamycin treatment in these transgenic mice was initiated prior to tumor onset (140 days). Two groups of 10 NDL2-5 transgenic mice were used for this study. The mice were randomized to receive either vehicle of rapamycin. Five mice were treated with rapamycin (5mg/kg/2xwk, i.p) and the other 5 were treated with vehicle starting at 140 days for 8 weeks. Since all the mice were not of same age, the treatments were staggered but all mice started to receive treatment at exactly 140 days. Mice were sacrificed at a mean age of 218 days old (range 204 to 234 days) because this was the average age where the control vehicle group had reached ethical endpoint and required euthanization. The results of this study are illustrated in the Kaplan-Meier Survival Curve as shown in Figure 3.12. Outcomes that were assessed were the following: *Date of Tumor Onset*: All 5 untreated vehicle mice developed palpable tumors at a mean of 168 days (range 155 to 183 days). Three of the rapamycin mice did not demonstrate any palpable tumors at study completion. The

Figure 3.12. Rapamycin delays the onset and progression of mammary tumors in activated Neu-expressing transgenic mice. Kaplan-Meier Survival Curve illustrating that rapamycin delays tumor onset and growth in Neu-activated transgenic mice. Ten NDL2-5 transgenic mice were treated with rapamycin (5mg/kg/twice weekly,i.p) or vehicle starting at 140 days for 8 weeks. Mice were sacrificed at a mean age of 218 days old (range 204 to 234 days). All 5 vehicle mice developed palpable tumor at a mean of 168 days (range 155 to 183 days). Three of the rapamycin mice did not demonstrate any palpable tumor at study completion. The remaining 2 developed palpable tumor at 181 and 219 days. When the 5 vehicle mice were sacrificed, all demonstrated obvious tumors greater than 10% of their body weight. Three of the rapamycin mice demonstrated no tumor, and the other two demonstrated minimal tumors


p < 0.01
Rapamycin
Control
% free of tumor
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0.0
0
10
20
30
40
50
60
70
80
Time since start of treatment

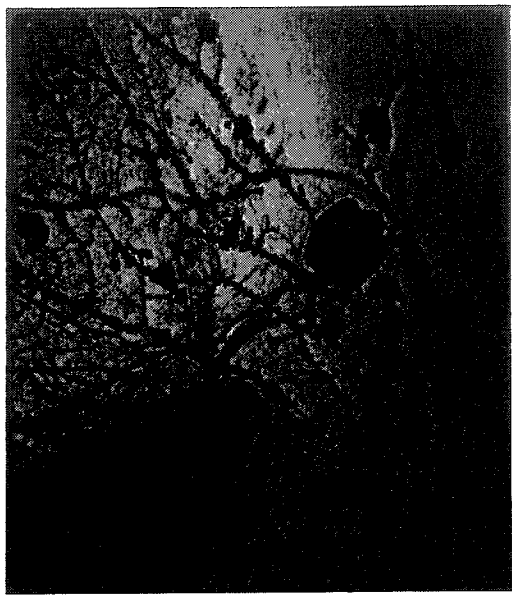
remaining 2 developed palpable tumors at 181 and 219 days. *Tumor Size*: When the 5 vehicle mice were sacrificed, all demonstrated obvious tumors greater than 10% of their body weight. Three of the rapamycin mice demonstrated no tumor, and the other two demonstrated minimal tumors. The results of this study demonstrate that NDL2-5 mice that receive regular treatment of rapamycin develop tumors with a longer latency period compared to control mice receiving vehicle treatment only. Rapamycin not only increases the time to event (ie detection of palpable tumors) but also decreases the rate of tumor growth when compared to untreated NDL2-5. The results of this study are the first to show the antitumor efficacy of rapamycin in an activated Neu-induced transgenic mouse model. Given the fact that many of the aberrant downstream targets of mTOR (eg. 4EBP1, S6K) and cell cycle regulatory proteins (eg. cyclin D1) in breast cancer are implicated in involvement of tumor initiation, suggests that mTOR may be an ideal target against which to develop therapeutics to prevent early tumor formation.

3.2.12 Rapamycin has an effect on the subgross and histological patterns of NDL2-5 transgenics

In addition to inhibition of tumor growth of NDL2-5 mice, rapamycin treatment also resulted in a decrease in the number of mammary tumors present. Consistent with previous studies examining the morphological patterns of NDL2-5 mammary glands, vehicle treated NDL2-5 mice exhibit large multifocal, proliferating tumors at 29 weeks of age (Figure 3.13A). Interestingly, mammary glands from rapamycin treated NDL2-5 mice of the same age display only diffuse cystic hyperplasias, lacking large focal proliferative lesions (Figure 3.13B). Histological analysis of hematoxylin/eosin stained sections derived from the mammary gland of NDL2-5 mice treated or untreated (vehicle) with rapamycin reveal that the vehicle mice show large, typical ErbB-type tumor (measuring 8 mm in dimension) which are clearly a carcinoma (Figure 3.14A). The rapamycin-treated glands on the other hand only show few small tumors measuring up to 3.5 mm in greatest diameter (Figure 3.14B). Importantly, rapamycin-treated mammary glands contain small clusters of ErbB-type mammary intraepithelial neoplasia (MIN) which are known to be precursors to invasive carcinoma. These mice also demonstrate

Figure 3.13. Wholemount analyses of mammary glands from vehicle versus rapamycin-treated Neu-expressing transgenic mice. Wholemount preparations of hematoxylin-stained mammary glands comparing the morphology of NDL2-5 mice treated and untreated with rapamycin. Note that the untreated NDL2-5 exhibit large multifocal, proliferating tumors whereas mammary glands from rapamycin treated NDL2-5 much smaller multifocal proliferating tumors.

A NDL2-5 Control (29 wks)



B NDL2-5 Rapamycin (29 wks)

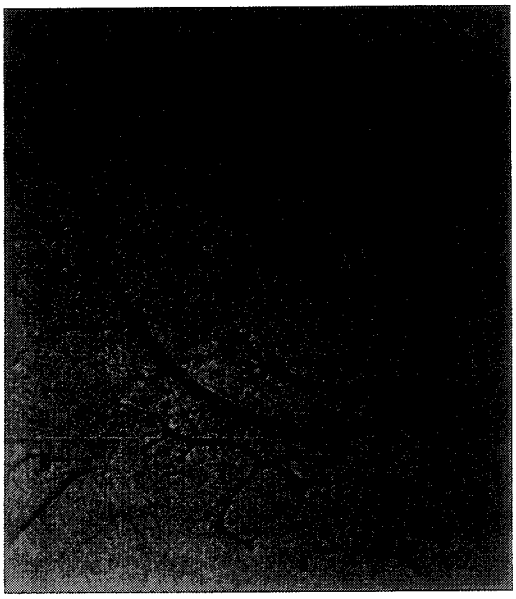
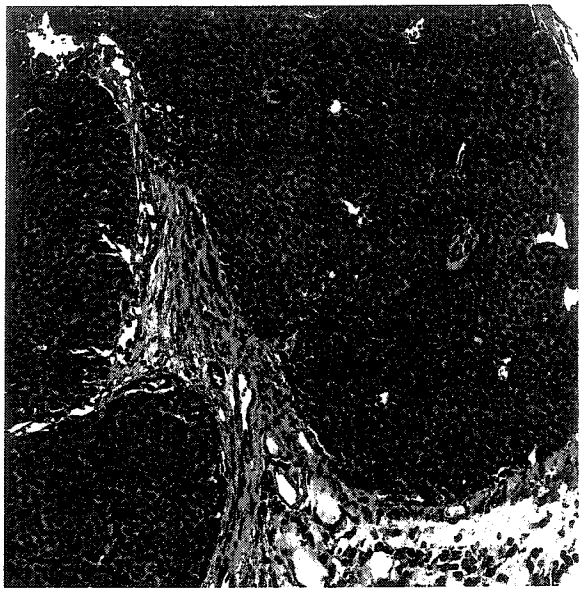


Figure 3.14. Histological analyses of mammary glands from vehicle versus rapamycin-treated Neu-expressing transgenic mice. Histological analysis of hematoxylin/eosin stained sections derived from the mammary gland of NDL2-5 mice treated or untreated (vehicle) with rapamycin. The vehicle mice show large, typical ErbB tumor measuring 8mm in greatest dimension, clearly a carcinoma. The rapamycin-treated glands display numerous small clusters of ErbB-type mammary intraepithelial neoplasia (precursors of invasive carcinoma).

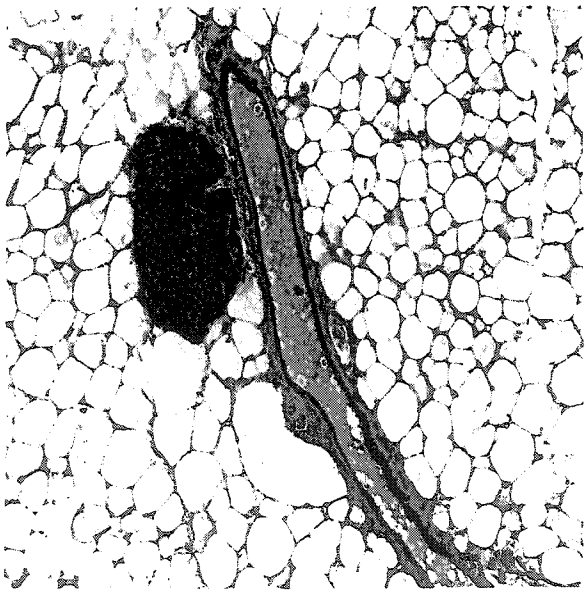
A

NDL2-5 Control (29 wks)



B

NDL2-5 Rapamycin (29 wks)

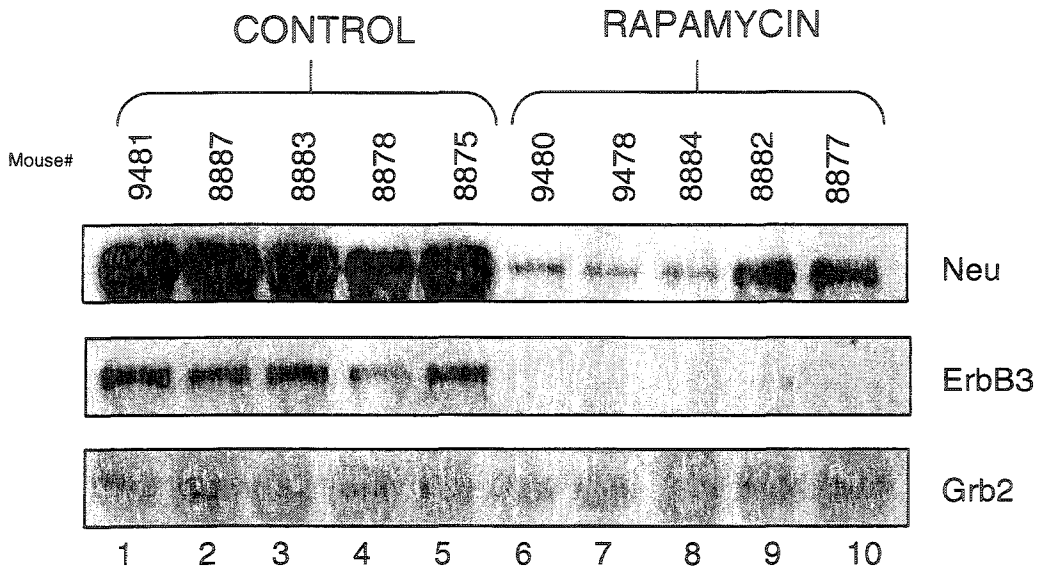


small glandular and solid acini that are scattered throughout the gland. These clusters have abnormal cells with poor orientation and larger nuclei with abundant cytoplasm (Figure 3.14B). The fact that rapamycin has an effect on MIN supports the notion that rapamycin increases the latency period in NDL2-5 mice.

3.2.13 NDL2-5 mice treated with rapamycin show decreases in ErbB2 and ErbB3 protein levels.

In order to determine the possible mechanism of rapamycin-induced inhibition of tumor growth in NDL2-2 transgenics, whole mammary glands were isolated from treated and untreated mice. The protein extracts were subjected to immunoblot analysis and levels of Neu, ErbB3 and downstream targets of mTOR were examined using specific antibodies. Figure 3.15 summarizes the findings. Consistent with previous studies, vehicle treated NDL2-5 mice show high levels of expression of the Neu transgene (Siegel and Muller, 1996). The two rapamycin-treated mice (8882 and 8877) that developed tumors prior to study completion have elevated levels of Neu but to a lower degree compared to the untreated group. Three of the 5 rapamycin-treated mice that do not develop palpable tumors at end of study, do not show upregulation of the Neu transgene. Previous studies have shown that the endogenous ErbB3 protein is upregulated in the tumors of these mice (Siegel et al., 1999). All 5 vehicle-treated mice in this study show expression of endogenous levels of ErbB3, however, analysis of all 5 rapamycin-treated mice reveal no expression of ErbB3 in their mammary glands. The two rapamycin-treated mice that developed tumors with longer latency before study endpoint also do not demonstrate increase in ErbB3 protein levels. This rapamycin-induced downregulation of ErbB3 strongly supports the role of mTOR in the regulation of ErbB3 in these tumors. Therefore, the inhibition of tumor growth compounded with the lack of ErbB3 expression in rapamycin-treated NDL2-5 mice suggests an important role for endogenous ErbB3 upregulation during Neu-mediated mammary tumorigenesis. This supports our hypothesis that ErbB3 may be providing the essential survival signals necessary for rapid tumor growth by coupling to the PI-3K/mTOR anti-apoptotic signaling pathway.

Figure 3.15. Neu-expressing transgenic mice treated with rapamycin show decreases in Neu and ErbB3 protein levels. Western immunoblot illustrating that rapamycin-treated NDL2-5 mice show decreases in Neu and ErbB3 protein levels. Equivalent amounts of total protein (50μg) from mammary tumor lysates were electrophoresed on a 4-12% gradient SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The blot was probed with either Neu or ErbB3-specific antibodies. Grb-2 immunoblots were used to confirm that equivalent amounts of protein were present in each lane. Note the lack of ErbB3 expression in rapamycin-treated mice.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3.2.14 Mammary glands of rapamycin-treated NDL2-5 mice show dephosphorylation of S6K and 4EBP1 but not Akt.

The phosphorylation status of S6K at two critical sites were examined. Interestingly, rapamycin-treated NDL2-5 mice show dephosphorylation of S6K on Thr 389 but not on Thr421/424 sites (Figure 3.16). Dephosphorylation of mTOR-specific and rapamycin-sensitive Thr 389 site suggests the importance of this phosphorylation site in Neu-mediated tumorigenesis. Note that the total levels of S6K protein remained unchanged in both groups.

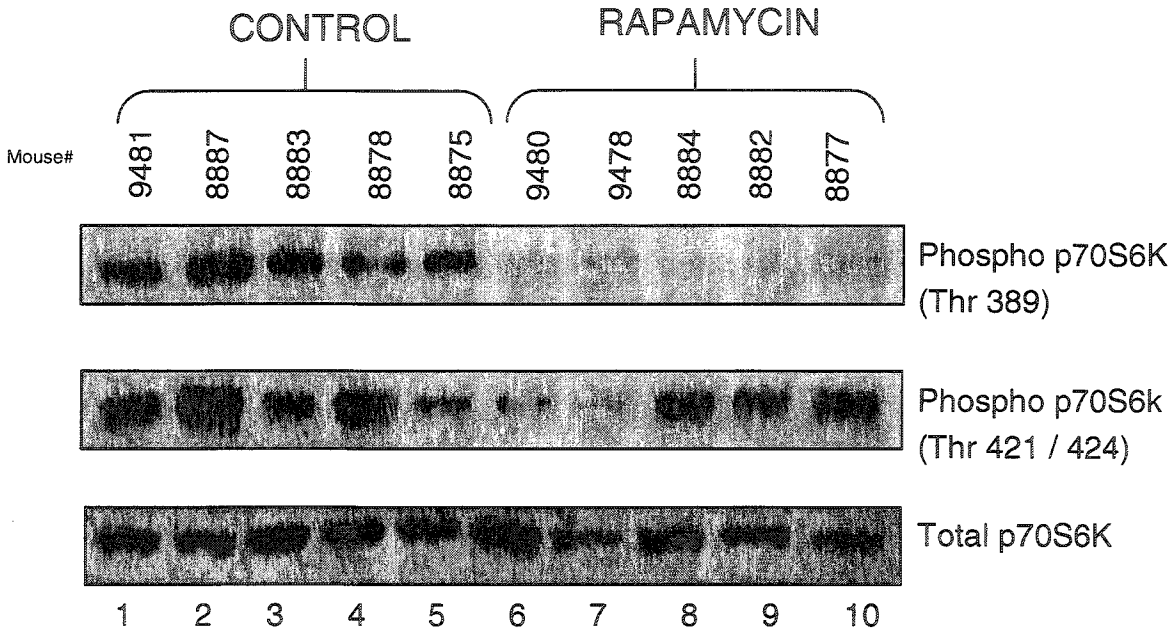
We also determined whether rapamycin-induced inhibition of tumor growth was a result of dephosphorylation of the mTOR effector target, 4EBP1. Rapamycin treatment of NDL2-5 mice led to complete dephosphorylation of 4EBP1 at the crucial Ser65 site (Figure 3.17). This strongly suggests that 4EBP1 is not dissociated but rather bound to eIF4E, thereby repressing translation of specific proteins. More interestingly, total 4EBP1 protein is elevated in mammary glands of rapamcyin treated NDL2-5 transgenics when compared to the vehicle treated mice. Therefore the end result of rapamycin treatment is an increase in hypophosphorylated 4EBP1 protein. This may suggest that rapamycin may be preventing tumor onset in NDL2-5 transgenics by maintaining abundant 4EBP1 in its active hypophosphorylated form and therefore inhibiting several proteins such as ErbB3.

As observed in tissue culture models, inhibition of mTOR in transgenic mice does not lead to dephosphorylation of Akt (Figure 3.18). This suggests Akt does not play a role in the rapamycin-induced inhibition of tumor growth in the NDL2-5 transgenic mouse model.

3.2.15 Mammary glands of rapamycin-treated NDL2-5 mice show a decrease in cyclin D1 mRNA and protein.

Earlier studies with NAFA tissue culture cells revealed that rapamycin induces G1 arrest but does not induce apoptotic cell death (Figures 3.8 and 3.9). Therefore, we hypothesized that the rapamycin-mediated inhibition of NDL2-5 tumor growth may be

Figure 3.16. Neu-expressing transgenic mice treated with rapamycin show dephosphorylation of S6K at the Thr389 site but not at Thr421/424. Western immunoblot illustrating that NDL2-5 mice treated with rapamycin show a decrease in S6K phosphorylation. Equivalent amounts of total protein (50μg) from mammary tumor lysates were electrophoresed on a 4-12% gradient SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The membrane probed with either phospho-specific S6K (Thr389 and Thr421/424) or S6K-specific rabbit polyclonal antibodies. Note that Thr 421/424 site on S6K remains unchanged whereas the mTOR-specific Thr 389 site is completely dephosphorylated. Also note that total S6K protein remains unchanged.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Phospho p70S6K
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Figure 3.17. Neu-expressing transgenic mice treated with rapamycin show an increase in total 4EBP1 protein levels but a decrease in its phosphorylation state (Ser65) compared to vehicle. Western immunoblot illustrating that NDL2-5 mice treated with rapamycin show a decrease in 4EBP1phosphorylation but an increase in its total protein levels. Equivalent amounts of total protein (50μg) from mammary tumor lysates were electrophoresed on a 4-12% gradient SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The membrane probed with either phospho-specific 4EBP1 (Ser 65) or 4EBP1-specific rabbit polyclonal antibodies. Note the decrease in phosphorylation status of 4EBP1 at the mTOR-dependent Ser 65 site but an increase in total 4EBP1 protein levels resulting in an increase in the activated form of the hypophosphorylated 4EBP1 in rapamycin-treated NDL2-5

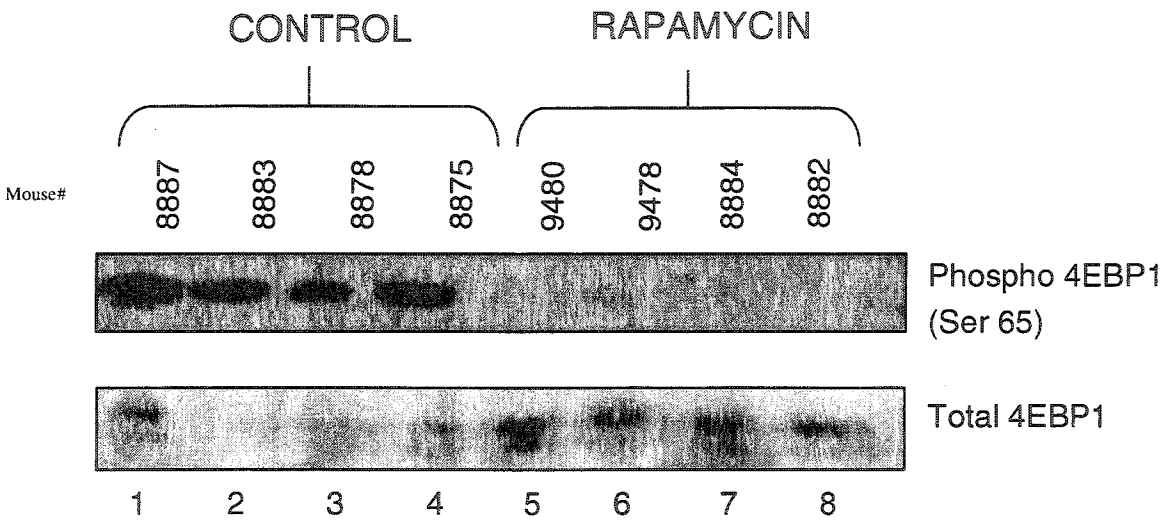
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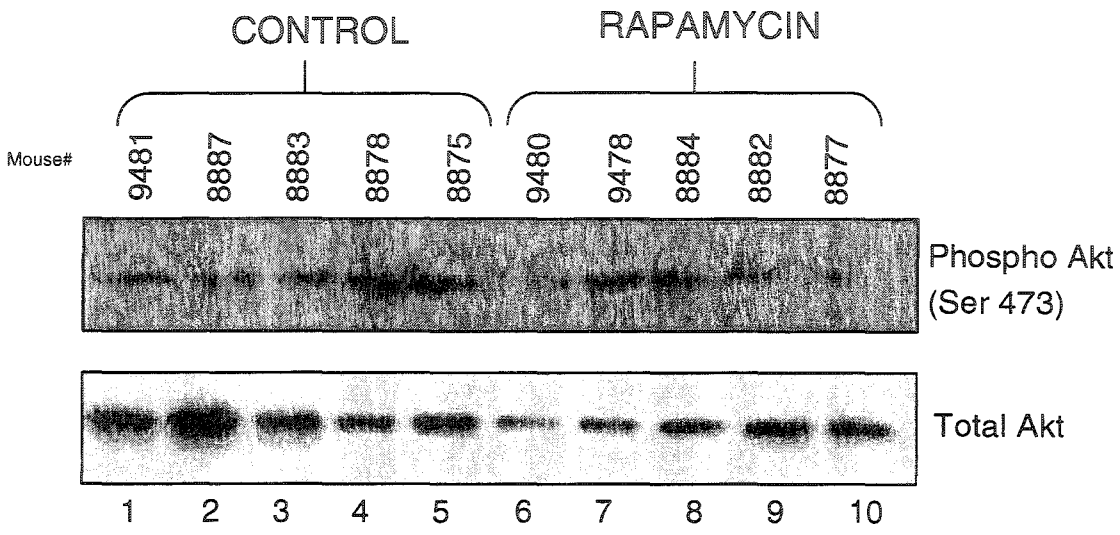
Figure 3.18. Neu-expressing transgenic mice treated with rapamycin do not show any changes in Akt phosphorylation when compared to vehicle. Western immunoblot illustrating that NDL2-5 mice treated with rapamycin do not show dephosphorylation of Akt. Equivalent amounts of total protein (50μg) from mammary tumor lysates were electrophoresed on a 4-12% gradient SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The membrane probed with either phospho-specific Akt or Akt-specific rabbit polyclonal antibodies. Note that Ser 473 site on Akt remains unchanged. Also note that total Akt protein remains unchanged.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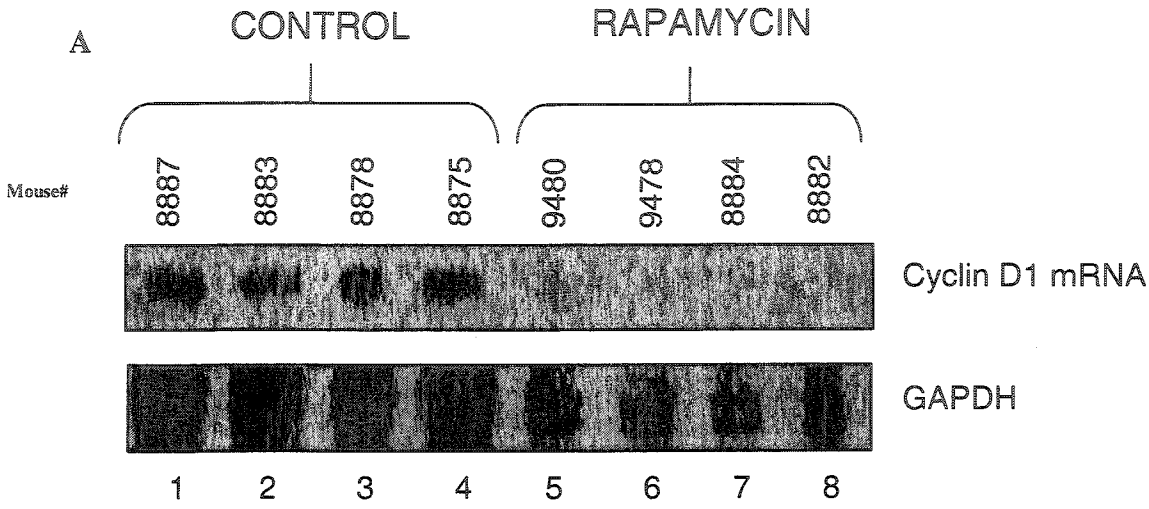
due to G1 cell cycle arrest. Previous studies illustrated that rapamycin-induced G1 arrest in NAFA cells correlated with downregulation of cyclin D1 levels both at the mRNA and protein levels (Figure 3.10). Therefore, we determined the levels of cyclin D1 in mammary glands of vehicle vs rapamycin-treated NDL2-5 mice. The results are shown in Figure 3.19. RNA was extracted from mammary glands and subjected to RNAse protection assay using cyclin D1-specific riboprobes. Figure 3.19A illustrates that rapamycin-treated NDL2-5 mice show a substantial downregulation of cyclin D1 at the mRNA level. Protein extracts were also isolated from the mammary glands of the two groups and subjected to immunoblot analysis using cyclin D1-specific antibodies. The results clearly show a decrease in cyclin D1 protein levels in rapamycin treated transgenics compared to vehicle (Figure 3.19B). These results may suggest that rapamcyin inhibits tumor growth by downregulating cyclin D1 which may then lead to arrest of mammary cells in the G1 phase of the cell cycle.

3.3 DISCUSSION

The results presented in this chapter provide a novel mechanism of ErbB3 regulation by PI-3K and mTOR during Neu-mediated mammary tumorigenesis. ErbB3, in conjunction with Neu, may be providing survival signals through activation of the PI-3K/mTOR/4EBP1 pathway. The antitumor activity of rapamycin in breast cancer may be mediated, in part, through the inhibition of the ErbB3 oncogene, therefore providing a molecular basis for the selective targeting of this signaling pathway in the treatment of HER2-mediated human breast malignancies.

One of the first and most striking results was the ability of mTOR to specifically regulate ErbB3 protein levels without affecting levels of Neu. There was a rapamycin-dependent decrease in ErbB-3 protein levels most likely as a result of increased ErbB-3 turnover only. Although the results in figure 3.7 clearly demonstrate that both PI-3K and mTOR are involved in increasing the stability of ErbB3 in Neu-induced tumors, it is not clear whether mTOR regulates ErbB3 at the translational level as well. Indeed, studies have shown that mTOR regulates cyclin D1 at both the translational and post-translational levels (Grewe et al., 1999; Hashemolhosseini et al., 1998).

Figure 3.19. Neu-expressing transgenic mice treated with rapamycin show a decrease in cyclin D1 mRNA and protein compared to vehicle. (A) RNase protection illustrating the decrease in cyclin D1mRNA in rapamycin-treated NDL-2-5 mice. Total RNA was isolated from these cells and levels of cyclin D1 transcripts were determined by RNase protection analysis. Total RNA (15 μg) extracted from mammary tumors were hybridized to an antisense cyclin riboprobe. The protected fragment corresponding to the cyclin D1 message is indicated. An antisense internal control probe directed against the mouse GAPDH gene was included to control for equal loading of RNA onto the gel. Normalization of cyclin D1 mRNA with GAPDH mRNA suggests a 50% decrease in cyclin D1 mRNA in rapamycin-treated mice compared to vehicle. **(B)** Western immunoblot illustrating levels of cyclin D1 protein in rapamycin-treated NDL2-5 mice. Equivalent amounts of protein (50 μg) from mammary tumor lysates were electrophoresed on a 4-12% gradient SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The blot was probed with cyclin D1-rabbit polyclonal antibodies. Grb-2 immunoblots were used to confirm that equivalent amounts of protein were present in each lane.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Cyclin D1 mRNA
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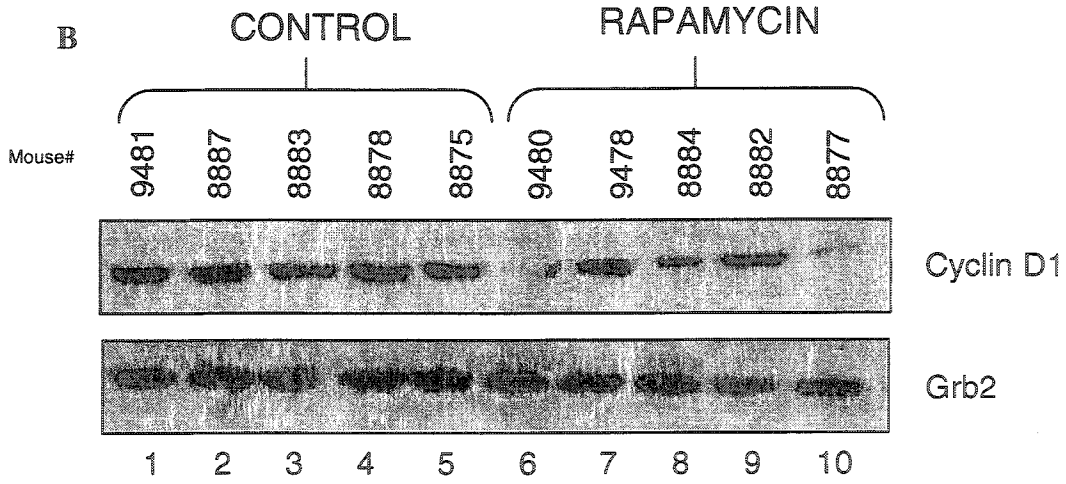
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Although the biochemical reason for specificity of mTOR for ErbB3 protein regulation was not determined, there are a few possibilities that may require further investigation. Indeed mTOR has been shown to link growth factor signaling and translational and post-translational control of several mammalian proteins (Peng et al., 2002; Rajasekhar et al., 2003; Shamji et al., 2000). Studies have suggested that mTOR is highly specific for the control of translation of 5'TOP (5'-terminal oligopyrimidine tract) mRNAs (Gingras et al., 1999b; Gingras et al., 2001a; Raught et al., 2001). It would be interesting to determine the differences in sequences within the 5'untranslated regions (UTR) of ErbB3 and Neu and to determine whether ErbB3 contains a stretch of 4-14 pyrimidines in its extreme 5'terminus region that may have been conserved for its regulation by mTOR. Another possibility may be that mTOR is not specific for 5'TOP mRNAs only and may therefore have specificity for ErbB3 for other unknown reasons. In support of this, recent studies have provided evidence to suggest that mTOR regulates the translation of the Ewing's sarcoma (EWS) protein as well as the EWS/FLI-1 fusion protein which is not translated from a 5'TOP mRNA (Mateo-Lozano et al., 2003). This study showed that the EWS/FLI-1-dependent upregulation of mTOR played an essential role in Ewing's sarcoma (ES) and that inhibition of mTOR with rapamycin (10 ng/ml) in cell culture for 24, 48 and 72 hours led to almost complete depletion of EWS and EWS/FLI-1 protein levels. Furthermore, expression of a rapamycin-resistant mTOR construct prevented both proliferation and decreases in EWS and EWS/FLI-1 protein levels.

Further evidence supporting mTOR-dependent regulation of ErbB3 comes from studies showing that mTOR regulates the expression of TGFβ type 2 receptor (TGFβ RII). In this study, treatment of Ewing's sarcoma cell lines with rapamycin (10 ng/ml) for 24 hours resulted in a dramatic increase in TGFβ RII at both the mRNA and protein levels (Mateo-Lozano et al., 2003). TGF- β has shown to be a potent inhibitor of mammary cell proliferation and overexpression of TGF- β in mouse mammary tissue induces mammary hypoplasia and inhibits tumorigenesis (Sherr, 1994; Sherr, 1996). Many types of cancer cells show partial or complete loss of the negative growth control by TGF- β which is thought to be an important step in the process of oncogenic transformation (Morgan, 1995). Interestingly, Siegel et al have recently shown that a

dominant negative TGFβ type II receptor decreases the latency of Neu-induced mammary tumors in transgenic mouse models suggesting the importance of TGFβ in impairing Neu-mediated transformation (Siegel et al., 2003). Therefore, downregulation of TGFβ may be another possible mechanism by which mTOR facilitates tumor progression. Similarly, since many ErbB3-overexpressing ErbB2 tumors may depend on ErbB3 for their full oncogenic potential, increasing ErbB3 protein levels may be another mechanism by which mTOR accelerates ErbB2-mediated tumor growth. Nonetheless, the above studies implicate mTOR in the regulation of a receptor and highlight the complexity of mTOR-dependent protein regulation.

Results of recent reports support mTOR-dependent post-translational regulation of ErbB3. These results come from proteomic studies on the specific translational control by rapamycin demonstrating that most proteins inhibited by rapamycin were RNA-binding proteins (Grolleau et al., 2002). Furthermore, translation of a set of genes that were unaffected by rapamycin treatment included transcription factors, kinases, phosphatases and members of the RAS superfamily. Since ErbB3 is a kinase, there remains the possibility that it may not be regulated at the level of translation by mTOR.

It is clear from results of figure 3.7 that both PI-3K and mTOR are regulating ErbB3 at the post-translational level in Neu-mediated tumors. In support of this finding, recent reports have linked mTOR with possible protein degradation pathways, however the mechanism of mTOR-dependent protein degradation remains unclear. Therefore, there is a possibility that although mTOR may not regulate the translation of ErbB3, it may regulate specific proteosomes involved in ErbB3 protein stability. For example, studies have shown that PI-3K/mTOR signaling inhibits the ubiquitin-mediated degradation of cyclin D1 (Diehl et al., 1998). Recent reports by Razeghi et al have also shown that mTOR inhibits the ubiquitin proteasome proteolytic (UPP) pathway, independent of Akt, in order to increase the half-life of some of its target proteins (Razeghi et al., 2003). Activation of the UPP pathway is associated with an increase in mRNA levels of ubiquitin, ubiquitin conjugating enzymes, ubiquitin ligases, and components of the proteasome (Bodine et al., 2001; Medina et al., 1995; Voisin et al., 1996). Inhibition of mTOR with rapamycin in skeletal muscles leads to decreased phosphorylation of S6K, 4EBP1 and an increase in the ubiquitin-dependent pathway of protein degradation

(Razeghi et al., 2003). Further studies supporting rapamycin-induced decrease in ErbB3 half-life comes from studies showing that the antibiotic benzoquinone ansamycin geldanamcyin induces degradation of the mTOR-regulated hypoxia inducible factor 1 alpha (HIF-1α) during treatment of prostate cancer (Mabjeesh et al., 2002). These studies support the possibility of mTOR increasing the stability of ErbB3 through inactivation of protein degradation pathways. Further studies will be necessary to investigate the mechanism responsible for PI-3K/mTOR-dependent regulation of ErbB3 stability during cellular transformation.

Rapamycin-induced dephosphorylation of S6K and 4EBP1 suggests that NAFA cells are sensitive to rapamycin. Inhibition of mTOR in the NAFA cell line leads to inactivation of S6K and activation of 4EBP1 so that it is hypophosphorylated and bound to the eIF4E. Although LY294002 and rapamycin lead to almost complete dephosphorylation of these two downstream targets of mTOR, comparable changes in ErbB3 protein levels were not observed. One possibility is that there may be alternative mTOR-independent pathway regulating ErbB3 protein that is unaffected by rapamycin. Another possibility is that the activated proteolytic system might not be sufficient to degrade the overexpressed and over-phosphorylated ErbB3 in these tumors.

Whether ErbB-3 is being regulated at the translational level remains to be determined. Nevertheless, the results presented here suggest that translational and/or post-translational control of ErbB-3 by PI3'K/mTOR may be important to mediate cell survival during ErbB-2-mediated tumorigenesis. The importance of ErbB3-dependent activation of PI-3K is further explored in the next chapter.

The ability of LY294002 and the dominant negative PI-3K to induce apoptosis strongly supports the hypothesis that PI-3K is a key mediator in sending the necessary anti-apoptotic signals during Neu-mediated transformation. The next chapter further supports this model in that both Neu and ErbB3 cooperate to activate the PI-3K pathway and induce cell survival. In support of this finding, other studies have shown that inhibition of PI-3K leads to apoptotic cell death (Davies et al., 2004; Webster et al., 1998).

The antitumor efficacy of rapamycin in Neu-induced tumors was indeed very promising. In the case of the transplanted NAFA isografts, rapamycin was far more

successful in preventing the onset and progression of tumors than it was in facilitating tumor regression. An explanation for this may be that when rapamycin is given the day of transplant, many of the cells arrest at the G1/S boundary earlier during tumor development and those cells that escape cell cycle inhibition contribute to the tumor size and demonstrate impressive growth inhibitory effects. The fact that rapamycin did not induce apoptosis may be one explanation for its inability to have a significant effect on tumor regression.

The most encouraging finding was the ability of rapamycin to increase the time of tumor onset and decrease the rate of tumor progression in the NDL2-5 transgenic mouse model. The mammary fat pad gland of rapamycin treated NDL2-5 show only small tumors measuring up to 1.5 mm in greatest diameter compared to 8 mm in control transgenics. The rapamycin treated mammary glands clearly show presence of small clusters of ErbB-type MIN which are precursors to invasive carcinoma (hyperplasias without atypia). The presence of MIN in rapamycin-treated mice strongly supports the finding that although rapamycin does not prevent tumor onset, it certainly delays the onset and progression.

One of the most striking results of the mouse clinical trial was the observation that ErbB3 levels were not detected in any of the NDL2-5 mice treated with rapamycin. Interestingly, the two rapamycin-treated mice that developed tumors prior to study completion, demonstrated upregulation of Neu without similar increases in ErbB3. Therefore, the rapamycin-mediated inhibition of ErbB3 may, in part, affect the latency period and the ability of overexpressed Neu to drive cell proliferation.

Interestingly, the transgenic mouse study also showed a significant increase in the levels of 4EBP1 in its active state in rapamycin treated NDL2-5. This strongly suggests that rapamycin increases the levels of hypophosphorylated (Ser 65) 4EBP1 in order to suppress the activity of various proteins including ErbB3 which is necessary for cell survival and tumor progression in NDL2-5 transgenics. In support, studies have shown that increased expression of 4EBP1 leads to reduced rates of cell growth and that transformed cells express higher levels of hyperphosphorylated 4EBP1. Furthermore, it has been shown that the Ras/MAPK pathway leads to increased phosphorylation of eIF4E, and the ability of Ras to transform cells is diminished when eIF4E expression is

decreased (Rinker-Schaeffer et al., 1992; Rinker-Schaeffer et al., 1993), implying that eIF4E and hence 4EBP1 proteins may be key mediators of Ras-mediated transformation. Although changes in 4EBP1 protein levels were observed, that was not the case with total S6K protein. In support of these findings, studies have shown that overexpression of eIF-4E has been reported in 100% of human breast cancers and that its overexpression by 7 fold is associated with poor prognosis (Li et al., 1997; Li et al., 1998). S6K on the other hand is amplified in less than 10% of human breast cancers and does not correlate with poor prognosis (Barlund et al., 2000). This finding raises the possibility that rapamycin inhibits tumor growth, in part, by increasing hypophosphorylated 4EBP1 and hence decreasing the ability of eIF-4E to bind to the 5'end of the mRNA.

Another interesting result from the transgenic mouse clinical trial studies was the rapamycin-induced dephosphorylation of S6K on the Thr 389 site only and not on the Thr 421/Ser 424 site. Studies have shown that although S6K has several phosphorylation sites, phosphorylation of Thr 389 is particularly important since substitution of this site with alanine inhibits its ability to phophorylate the S6 protein on the small ribosomal unit. Furthermore, Thr 389 site is the only site directly phosphorylated by mTOR (Dennis et al., 1996). Therefore, our findings suggest that phosphorylation of Thr 389 may be crucial for ErbB2-mediated mammary tumor progression.

The results in this chapter further show that in addition to its inhibitory effects on S6K and 4EBP1, rapamycin also interfered with a crucial member of the cell cycle machinery. Rapamycin-treated NAFA cells as well as rapamycin-treated NDL2-5 mice demonstrated a decrease in cyclin D1 both at the mRNA and protein levels. In support of this finding, it has been suggested that mTOR plays an important role in the regulation of cyclin D1 (Grewe et al., 1999; Hashemolhosseini et al., 1998). The mechanism of mTOR-induced cyclin D1 regulation is unclear, however, it has been proposed that rapamycin delays accumulation of cyclin D1 mRNA during G1 and affects its mRNA stability and accelerates the degradation of newly synthesized cyclin D1 protein (Hashemolhosseini et al., 1998; Muise-Helmericks et al., 1998). Furthermore, inhibition of mTOR by the rapamycin analog (CCI-779) results in the reduction of cyclin D1 expression in MDA-468 breast cancer cell line (Yu et al., 2001a). These actions result in a decrease in the translation of cyclin D1 mRNA caused by inhibition of 4EBP1 which

results in a deficiency of active cyclin-dependent kinases (CDK)4/cyclin D1 complexes required for Rb phosphorylation. Our results suggest that these actions, along with the inhibition of ErbB3 may be resulting in tumor growth inhibition (Noh et al., 2004). Indeed, recent studies have suggested that although overexpression of PI-3K, Akt and S6K are evaluated as predictors of rapamycin-sensitivity, it is cyclin D1 levels that provide the marker for response to rapamycin-induced tumor inhibition. In support, studies have shown that overexpression of cyclin D1 has been reported in 50% of invasive breast cancers and is associated with tumor progression (Buckley et al., 1993; Weinstat-Saslow et al., 1995). These findings suggest that inhibition of cyclin D1 may very likely contribute to the antiproliferative activities of rapamycin and mTOR may be involved in coupling the growth stimulus provided by ErbB3 and cell cycle progression facilitated by cyclin D1. The next chapter demonstrates that cyclin D1 expression is partly dependent on the ability of ErbB3 to recruit and activate the PI-3K/mTOR pathway. Since cyclin D1 depends on this pathway for its upregulation, ErbB3 may play an important role in activating and sustaining the high levels of cyclin D1 which has been shown to be required for Neu-mediated transformation (Lee et al., 2000).

Studies have suggested several mechanisms by which rapamycin inhibits tumor growth. Inhibition of cdks, S6K, 4EBP1, Rb and acceleration of cyclin D1 turnover all contribute to its inhibitory effects. Recently, Guba et al. have shown that rapamycin inhibits tumor growth in mouse models through the inhibition of tumor vascularization correlating with a significant decrease in VEGF production (Guba et al., 2002). Similarly our results demonstrate that although downregulation of ErbB3 alone does not contribute to the rapamycin-induced tumor inhibition, it may certainly represent one possible novel antitumor mechanism of rapamycin, specifically in those tumors that depend on ErbB3 for proliferation.

One finding in this thesis refutes an important aspect of the hypothetical model. The model suggests that Akt is the mediator between ErbB3/PI-3K and mTOR signaling. Generation of this hypothesis was based on previous published results showing that PI-3K sends anti-apoptotic signals predominantly through Akt activation (reviewed in (Krasilnikov, 2000). Akt levels, however, remained unchanged in all cell culture experiments as well as in the animal studies suggesting that it is not involved in the

phosphorylation of S6K/4EBP1 and activation of ErbB3. More interestingly, inhibition of PI-3K did not lead to dephosphorylation of Akt at the Ser 473 site. One possibility is that the NAFA cell line may express a constitutive active form of Akt which is not affected by PI-3K inhibition. Nonetheless, there are a few recent studies that propose Akt-independent mechanisms of PI-3K and mTOR regulation which may also explain the findings in this thesis.

Although PI-3K-dependent activation of S6K and 4EBP1 was predominantly believed to occur via Akt, recent evidence shows that PI-3K can activate S6K independently of Akt through an alternative pathway involving PDK1 (Radimerski et al., 2002). Akt directly phosphorylates a number of proteins that impact cell survival and proliferation, however, the details defining the connection between PI-3K, Akt and mTOR are still unclear. As mentioned in chapter 1, a series of biochemical and genetic studies have revealed a pathway from Akt to mTOR involving the tuberous sclerosis complex (TSC) proteins tuberin and hamartin, as well as the small Ras-like GTPase, Rheb (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002). Recent identification of these novel regulators of mTOR implicate the complexity of this growing pathway. There is a possibility that there may be a direct link between PI-3K and TSC2 or Rheb which would allow Akt independent activation of mTOR. Nonetheless, the simple linear model of ErbB3/PI-3K/Akt/mTOR cannot account for these experimental findings, therefore, further studies are required to elucidate the connection between ErbB3 and mTOR targets.

Another possible component of the PI-3K pathway that may be involved in linking ErbB3 to mTOR may be TSC2. Although recent studies have focused on the importance of Akt in the phosphorylation and inactivation of TSC2, others have suggested that TSC2 may be altered by a novel pathway that involves post-translational modification of this protein that prevents it from inhibiting Rheb (Dan et al., 2002; Plas and Thompson, 2003). It would be interesting to determine whether ErbB3 has an effect on the TSC1/TSC2 complex that is independent of Akt. There is a possibility that cells that overexpress ErbB3, depend on ErbB3 to disrupt the TSC1/TSC2 complex. Future experiments may involve targeting endogenous ErbB3 without affecting ErbB2 signaling (Holbro et al., 2003) and assessing the phosphorylation status of TSC2 and Rheb.

Another explanation for why Akt levels may not be altered upon disruption of the ErbB3/PI-3K/mTOR pathway may be that the antibodies used in the western blot analyses were specific for Akt1 only. Indeed it has been shown that Akt2 and Akt3 are frequently upregulated in HER-2/neu-positive positive breast cancers and that it may contribute to tumor aggressiveness by enhancing cell survival (Bacus et al., 2002; Cheng et al., 1996; Nakatani et al., 1999). Further experiments are required to determine the phosphorylation statuses of different Akt isoforms and whether they are affected by inhibition of ErbB3/PI-3K interaction and/or by inhibition of PI-3K and mTOR.

From a clinical point of view, CCI-779 (analog of rapamycin) has reached phase II of development in clinical trials (Banerji et al., 2001; Chan et al., 2002). Phase I single-agent trials showed potential activity in breast cancer. The results of the Phase I/II clinical trials suggest that patients with advanced disease do not respond favourably to the drug. The results in this chapter, suggest that this finding is not surprising since rapamycin was not successful in reduction of tumor growth at later stages of tumor development. The ability of rapamycin to prolong tumor onset and progression in mouse tumor models suggests that patients with less advanced cancer may respond more favourably. Therefore, use of rapamycin in clinical trials may reveal that it is better at stabilizing the disease and preventing it from progressing versus reducing the disease burden. One potential reason for the inability of rapamycin to be as effective in tumor regression may be due to the fact that the molecular abnormality (overexpression of ErbB3-dependent PI-3K pathway) may represent a late event in the history of the tumor. Indeed the disease stage could be critical since inhibitors that block an initiating oncogenic event are, presumably, more likely to induce a clinical response than the ones that block a later event involved in disease progression.

In addition to defining maximal therapeutic indices, an important challenge during clinical development includes the ability to predict which tumors will be sensitive or resistant to rapamycin. Therefore, a better understanding of the possible mechanisms by which rapamycin acts as an antitumor agent may enable targeting of patients that will most favourably respond to treatment. Indeed Yu et al. have demonstrated that breast cancer cell lines found to be remarkably sensitive to rapamycin overexpress ErbB2 and possess constitutively activated PI3'K/mTOR pathway (Yu et al., 1998). However, the

reason for this sensitivity to rapamycin remains unclear. The results of this chapter suggest that inhibition of ErbB3 activity may be critical for downregulation of the constitutively activated PI-3K/mTOR pathway and this may represent one mechanism by which rapamycin induces its anti-proliferative effects. These results provide a rational or a model for why many ErbB2 overexpressing tumors depend on rapamycin-sensitive pathways for growth and are sensitive to rapamycin and its analogs.

CHAPTER 4

ErbB3 drives Neu-mediated transformation by coupling to PI-3K and activating the mTOR/4EBP1 pathway.

4.1 INTRODUCTION

The activity of ErbB2 in normal cells depends on dimerization with another ligand-binding ErbB receptor. However, in tumor cells, amplification of c-ErbB2 results in dramatic overexpression and constitutive activation of the receptor. A wealth of clinical data has demonstrated that ErbB2 overexpression plays a role in the development of breast cancer. ErbB2 overexpresion is observed in 25-30% of breast cancer and correlates with poor prognosis of the patient (King et al., 1985; Slamon et al., 1987; Slamon et al., 1989; Yokota et al., 1986). Interestingly, overexpression of phosphorylated ErbB3 has been observed in many tumors that overexpress ErbB2. Since ErbB3 itself has impaired tyrosine kinase activity, it needs a dimerization partner to become phosphorylated and hence acquire signaling potential. The most likely heterodimerization partner is ErbB2 since it is overexpressed in ErbB2-mediated tumors.

The significance of ErbB2 (Neu) and ErbB3 co-expression in both human and transgenic mammary tumors is not well understood. One potential explanation is that both ErbB2 and ErbB3 recruit distinct yet complimentary signaling pathways that cooperate during mammary tumor progression. Studies have shown that ErbB2 sends strong proliferative signals predominantly through the Ras/MAPK pathway. Perhaps the role of ErbB3 in these tumors is to provide cell survival signals by recruiting the p85 regulatory unit of PI-3K and activating mTOR and its downstream targets. Indeed it has been shown that ErbB3 contains six docking sites for the p85 regulatory subunit of PI-3K and it efficiently couples to the PI-3K-mediated pathway (Fedi et al., 1994; Prigent and Gullick, 1994). The hypothesis of this chapter is that both ErbB2-dependent proliferative signals and ErbB3-dependent cell survival signals are necessary for complete transformation. In support of this hypothesis, studies have shown that cell proliferation and cell survival are distinct yet coupled processes that need to go hand in hand to induce

cellular transformation (Amundadottir et al., 1996). One example supporting the importance of cell survival pathways during transformation derives from studies using transgenic mice overexpressing a mutant PyVmT (mT) antigen (Y315/322F) uncoupled to the PI-3K pathway (Webster et al., 1998). These mice develop apoptotic epithelial hyperplasias compared to wild-type mT antigen-expressing transgenics that develop multi-focal metastatic mammary tumors (Guy et al., 1992b). Interestingly, tumors arising from these hyperplasias have elevated levels of both endogenous ErbB2 and ErbB3 protein levels (Webster et al., 1998).

The focus of this chapter was to determine whether ErbB3 tyrosine phosphorylation is a mere consequence of ErbB2 activation and signaling or whether it is a necessary step to activate the PI-3K/mTOR pathway and thus contribute directly to the proliferation of mammary tumors. Furthermore, this chapter determines whether inhibiting the activity of Neu affects transformation and subsequent inhibition of ErbB3 as well as the PI-3K/mTOR pathway. The remainder of this section summarizes the findings of this chapter.

Neu activation was inhibited by use of an adenovirus expressing the Neu protein with an inactive kinase domain (NeuKD). The NeuKD (Neu Kinase Dead) receptor can still homo-or heterodimerize with Neu and ErbB3, respectively but loses its ability to auto- and transphosphorylate. The results show that NeuKD completely blocks Neu-mediated transformation of Rat-1 fibroblasts and this correlates with inactivation of ErbB3 as well as the targets of mTOR (S6K, 4EBP1 and cyclin D1). So far, the evidence provided suggests only a possibility that downregulation of ErbB3 may, in part, play a role in the decreased proliferation and inactivation of the mTOR/4EBP1 pathway.

In order to determine whether inhibition of proliferation and inactivation of mTOR targets was, in part due to inhibition of ErbB3/p85 interaction, a mutant ErbB3 (ErbB3-6F) was expressed in Neu-mediated tumor cells. This mutant ErbB3 has mutations in all six of its p85 docking sites that prevents it from interacting with and activating PI-3K. This allows for direct assessment of the importance of ErbB3 in activating PI-3K and subsequently sending signals that govern mTOR-dependent cell cycle regulation as well as PI-3K-dependent antiapoptotic signals. The results presented in this chapter demonstrate that both Neu and ErbB3 heterodimerize to function as an

oncogenic unit. Neu requires ErbB3-dependent activation of PI-3K/mTOR pathway for its full oncogenic potential. Importantly, ErbB3-6F mutant induces apoptosis in Neu-mediated tumors suggesting that these tumors highly depend on ErbB3's ability to recruit PI-3K in order to induce the survival signals necessary for complete transformation. Finally, the ErB3-6F mutant prevents the growth of tumors *in vivo* and also facilitates tumor regression.

Taken together, these results suggest that ErbB2 and ErbB3 heterodimerize in order to function together as a unit. Neu requires ErbB3 to activate the PI-3K/mTOR pathway and to accelerate mammary tumor cell proliferation.

4.2 RESULTS

4.2.1 NeuKD inhibits transforming activity of activated Neu in rat-1 fibroblasts

Activation of ErbB-3 during Neu-induced mammary tumorigenesis most likely results from transmodulation by constitutively activated Neu. Studies have proven that Neu sends strong proliferative signals predominantly through the Ras/MAPK pathway. Members of the Ras/MAPK pathway have shown to regulate mTOR mediators (Blalock et al., 2003; Gao et al., 2003; Kelleher, III et al., 2004). For example the MEK inhibitor PD-98059 significantly dephosphorylates S6K at Thr 421/Ser 424 (Lehman et al., 2003). On the contrary, other studies have suggested that Ras/MAPK and PI-3K/mTOR pathways are independent (Pene et al., 2002). According to the hypothetical model, kinase inactive ErbB3 depends on Neu for its ability to activate the PI-3K/mTOR pathway. Therefore, we assessed whether inactivation of Neu and the subsequent inhibition of the Ras/MAPK pathway leads to a decrease in the mTOR/4EBP1 pathway and a subsequent decrease in ErbB-3 protein levels. An adenovirus expressing the dominant negative form of Neu (NeuKD) was used to inhibit the function of Neu in NAFA cells. NeuKD possesses a mutation in its kinase domain which prevents its ability to auto and transphosphorylate. However, this NeuKD receptor retains its ability homo and heterodimerize with other EGFR members.

We first determined the ability of NeuKD to inhibit Neu-mediated transformation. This was determined by performing focus forming assays in Rat-1 fibroblasts using point-activated Neu (NeuNT). An adenovirus expressing the kinase-dead Neu mutant (AdNeuKD) was used to infect Rat-1 fibroblasts expressing the NeuNT gene. Adlacz was used as a positive control to ensure that any changes observed were due to the expression of NeuKD alone and not due to the presence of an adenovirus. The results are summarized in Table 4.1 and they clearly demonstrate that NeuKD has a dramatic effect on NeuNT-induced transformation. The average number of foci per plate in NeuNT-induced transformation is 158±15. Addition of AdNeuKD completely inhibited foci formation. Addition of Adlacz had no effect on the number of foci suggesting that inhibition of transformation was not due to a viral effect. Two additional activated oncogenes (middle T antigen and RasV12) were used as controls to determine whether the inhibition of NeuNT transformation by NeuKD was specific to Neu-mediated transformation. Interestingly, NeuKD did not have any effect on the transformation of Rat-1 fibroblast by either middle T antigen or RasV12. These studies strongly demonstrate that NeuKD is able to inhibit Neu-induced transformation. Further biochemical analysis (described below) may allow for understanding of the molecular mechanism by which NeuKD may be inhibiting transformation.

4.2.2 NeuKD downregulates Neu and ErbB3 in NAFA cells

In order to determine whether NeuKD has an effect on ErbB3/PI-3K/mTOR pathway, NAFA cells were infected with AdNeuKD and a series of biochemical analyses were performed. One of the most interesting results is that NeuKD decreases total ErbB3 protein levels as shown in Figure 4.1. This suggests that Neu itself is involved in regulating ErbB3 protein levels. Since previous results suggest that the PI-3K/mTOR pathway may be regulating ErbB3 at the translational or post-translational level, there may be a possibility that Neu may be exerting its action on ErbB3 by being involved in the regulation of S6K and 4EBP1. Described below (section 4.2.4) are the phosphorylation statuses of these mTOR effectors in the presence of NeuKD.

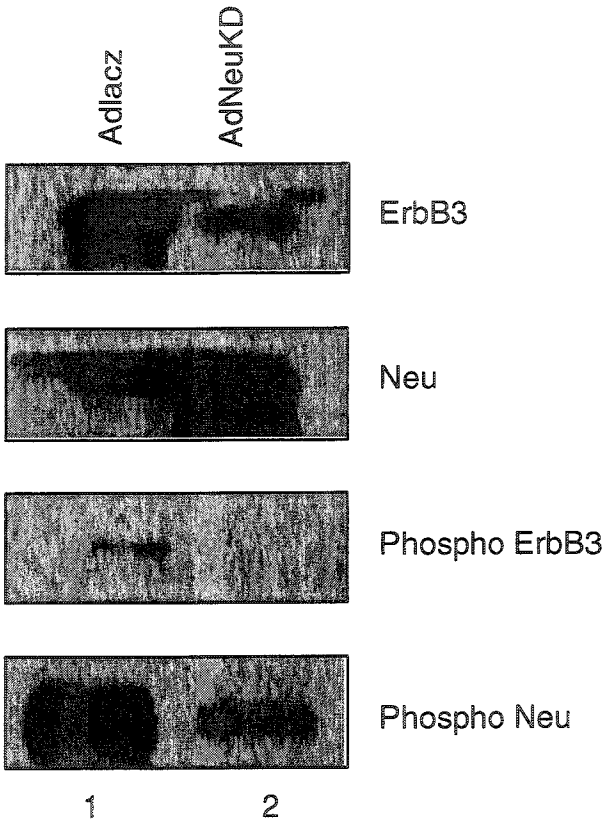
Table 4.1 NeuKD inhibits the transforming activity of activated Neu in rat-1 fibroblasts. Results of focus forming assays demonstrating that an adenovirus expressing the dominant negative form of Neu (NeuKD) inhibits Neu-induced transformation in Rat-1 fibroblasts. Focus assays were performed with point-activated Neu (NeuNT). Middle T antigen (MT) and constitutively activated Ras (RasV12) were used as controls to show that NeuKD specifically inhibits Neu-mediated transformation. Foci were scored over 6 plates. Note that NeuNT-mediated transformation is completely inhibited by infection of AdNeuKD while MT and RasV12-mediated transformation is not affected by AdNeuKD.

Table 4.1 Effect of AdNeuKD on transformation of Rat-1 fibroblasts transfected with either activated Neu (NeuNT), middle T antigen (mT) or Ras (RasV12).

Expression Plasmid	Average No. of foci/plate
pJ4ΩNeuNT	158 ± 15
pJ4ΩNeuNT + Adlacz	161 ± 22
pJ4ΩNeuNT + AdNeuKD	0
pJ4ΩmT	214 ± 17
pJ4ΩmT + AdNeuKD	216 ± 13
pJ4ΩRasV12	184 ± 21
pJ4ΩRasV12 + AdNeuKD	188 ± 18

All cDNAs are placed under the transcriptional control of the Moloney murine leukemia virus long terminal repeat (Mo-MuLV LTR). Rat-1 fibroblasts were maintained in a monolayer for 14 days following electroporation at which time they were stained with Geimsa. Values for each assay represent mean number of foci/plate counted on three plates ± standard deviation. This experiment was repeated three times with similar results.

Figure 4.1 NeuKD decreases ErbB3 protein levels and dephosphorylates Neu and ErbB3 in NAFA cells. Western blot illustrating that infection of NAFA cells with AdNeuKD leads to a decrease in ErbB3 protein levels as well dephosphorylation of ErbB3 and Neu. Equivalent amounts of protein lysates (50 μg) from AdNeuKD-infected NAFA cells were electrophoresed on a 4-12% gradient SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The membrane was probed with either ErbB-3-specific rabbit polyclonal antibodies. Equivalent amounts of lysates (1500 μg) from AdNeuKD-infected NAFA cells were also immunoprecipitated with phospho-tyrosine antibodies (P-tyr) and blotted for either Neu or ErbB3 using Neu or ErbB3-specific antibodies. Note the decrease in ErbB3 total protein as well as a dephosphorylation of ErbB3 and Neu upon AdNeuKD infection.

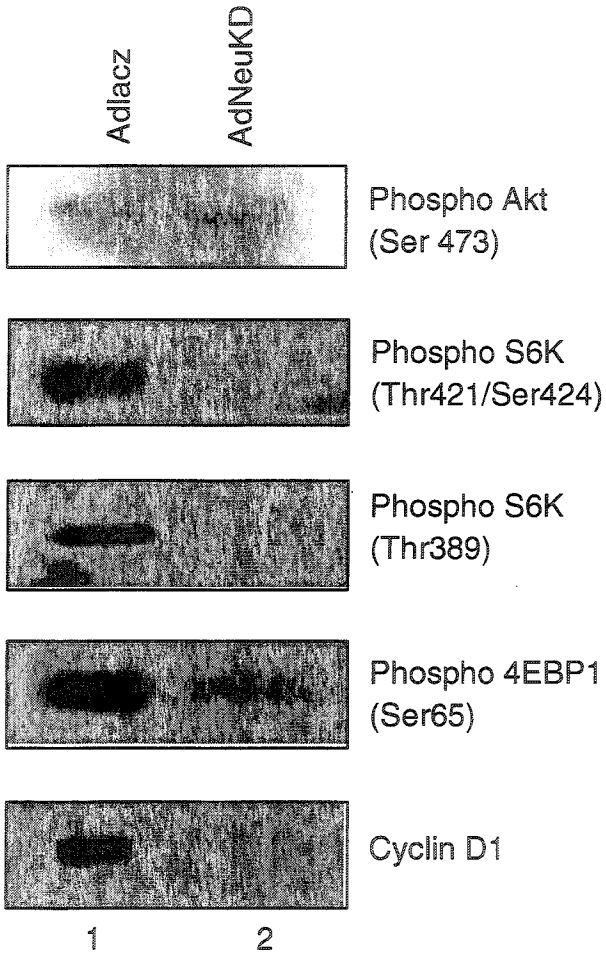

Adlacz
AdNeuKD
ErbB3
Neu
Phospho ErbB3
Phospho Neu
1
2

As mentioned earlier, NeuKD lacks the ability to auto and transphosphorylate other EGFR members. Since ErbB3 depends on Neu for it phosphorylation and activation, we investigated the effects on NeuKD on ErbB3 phosphorylation state. The results in Figure 4.1 show a substantial dephosphorylation of Neu upon infection with AdNeuKD. Notice that Adalcz had no effect on Neu phosphorylation. Furthermore, the levels of Neu protein are expectedly higher since the antibody cannot distinguish between the wild type and mutant forms. A dramatic increase in Neu protein levels upon AdNeuKD treatment confirms the high efficiency of the infection. Dephosphorylation of Neu upon NeuKD infection confirms that NeuKD is unable to phosphorylate itself. Interestingly, NeuKD also lead to dephosphorylation of the ErbB3 protein. This strongly supports the hypothesis that Neu is the predominant trans-modulator of ErbB3 in Neu-induced tumors.

4.2.3 NeuKD leads to dephosphorylation of S6K, 4EBP1 and inactivation of cyclin D1 protein.

As shown above, NeuKD decreases levels of ErbB3 protein. Results of this thesis also suggest that S6K and 4EBP1 may be involved in the translational and/or post-translational regulation of ErbB3. Therefore, in order to determine whether Neu is involved in the regulation of ErbB3 protein by exerting its action on the downstream mTOR effectors, we determined the phosphorylation statuses of S6K and 4EBP1 after inhibition of Neu activity. The results are shown in Figure 4.2. Interestingly, NeuKD leads to dephosphorylation of 4EBP1 at the Ser 65 site. This suggests that 4EBP1 is activated and is bound to the eIF4E thereby preventing translation of specific proteins. Furthermore, S6K is dephosphorylated at Thr389 suggesting that the S6 ribosomal protein is not phosphorylated, therefore, preventing mRNA binding. Interestingly, NeuKD also has a dramatic effect on cyclin D1 protein levels. NeuKD, however, did not affect the phosphorylation status of Akt. This refutes the hypothetical model and suggests that Akt may not play an important role in tumor progression in this cell line. These results strongly suggest that activated Neu plays an important role in regulating the mTOR pathway in these tumors.

Figure 4.2. NeuKD inhibits S6K, 4EBP1 and cyclin D1 in NAFA cells. Western blot illustrating that infection of NAFA cells with AdNeuKD leads to dephosphorylation of S6K/4EBP1 and a substantial decrease in cyclin D1 protein levels. Equivalent amounts of protein lysates (50 μg) from AdNeuKD-infected NAFA cells were electrophoresed on a 4-12% gradient SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The membrane was probed with either phospho-specific S6K (Thr 421/424 and Thr389), phospho-4EBP1-specific (Ser65) or cyclin D1-specific rabbit polyclonal antibodies. Dephosphorylation of S6K at both Thr422/Ser424 and Thr 389 was observed along with a decrease in 4EBP1 phosphorylation at Ser 65 site. Cyclin D1 protein levels also decrease upon AdNeuKD infection of NAFA cells.


Adlacz
AdNeuKD
Phospho Akt
(Ser 473)
Phospho S6K
(Thr421/Ser424)
Phospho S6K
(Thr389)
Phospho 4EBP1
(Ser65)
Cyclin D1
1
2

4.2.4 NeuKD induces apoptosis in NAFA cells.

Interestingly, NeuKD leads to apoptotic cell death (Figure 4.3) as observed by TUNEL assay. Since the hypothetical model illustrates the coupling of Neu/Ras/MAPK pathway to PI-3K/mTOR pathway, these results support the model and strongly suggest that the Neu/MAPK pathway is coupled to cell survival pathways. Taken together these results suggest that PI-3K in conjunction with Neu may be providing the necessary survival or anti-apoptotic signals during Neu-mediated transformation.

4.2.5 ErbB3-6F mutant cannot bind to PI-3K but can still interact with Shc.

As mentioned earlier, the hypothetical model illustrates that ErbB3 provides survival signals by coupling to the SH2 bearing p85 regulatory unit of PI-3K and subsequently activating mTOR and its targets. If this theory holds true, then inhibiting the interaction of ErbB3 and PI-3K should result in downregulation of members of this antiapoptotic pathway and lead to induction of apoptosis. The next couple of sections provide results supporting this hypothesis. An adenovirus expressing a mutant ErbB3 (ErbB3-6F or Ad6F) unable to bind to and activate PI-3K was generated in order to study the effects of ErbB3-PI-3K interactions in Neu-induced tumors. As mentioned earlier, this mutant contains a phenylalanine group instead of tyrosine in each of the 6 YXXM motifs that are specifically recognized by the SH2 domain located in the p85 subunit of PI-3K. We assessed the biochemical properties of ErbB3-6F mutant by infecting NAFA cells with Ad6F. An adenovirus expressing the β-galactosidase gene (lacz) was used as a control to ensure that changes observed were due to the presence of the ErbB3-6F mutant alone. Figure 4.4A shows that there is a dramatic decrease in the ability of ErbB3-6F to bind to p85. In order to confirm that the substitution of the six tyrosine residues did not alter the conformation of the C-terminal domain of ErbB3 such that the Shc binding site was less accessible, we confirmed whether the ErbB3-6F mutant is capable of recruiting Shc. The results indicate that the ErbB3-6F mutant still retains its ability to bind to Shc through its NPXY motif located in the carboxyl terminus.

Figure 4.3. NeuKD induces apoptosis in NAFA cells. TUNEL analysis showing induction of apoptosis in NAFA cells after 72 hr treatment with NeuKD. NAFA cell lines were infected with either control Adlacz virus or AdNeuKD followed by TUNEL assay after 72 hrs. NeuKD leads to apoptotic cell death whereas cells left untreated or infected with Adlacz do not show signs of apoptotic cell death. Cells undergoing apoptosis have their nucleus stained dark brown.

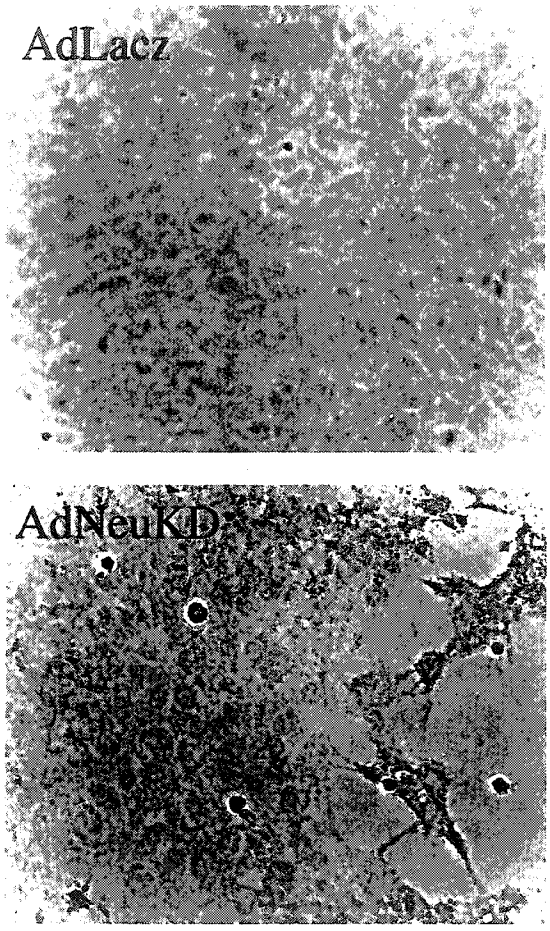
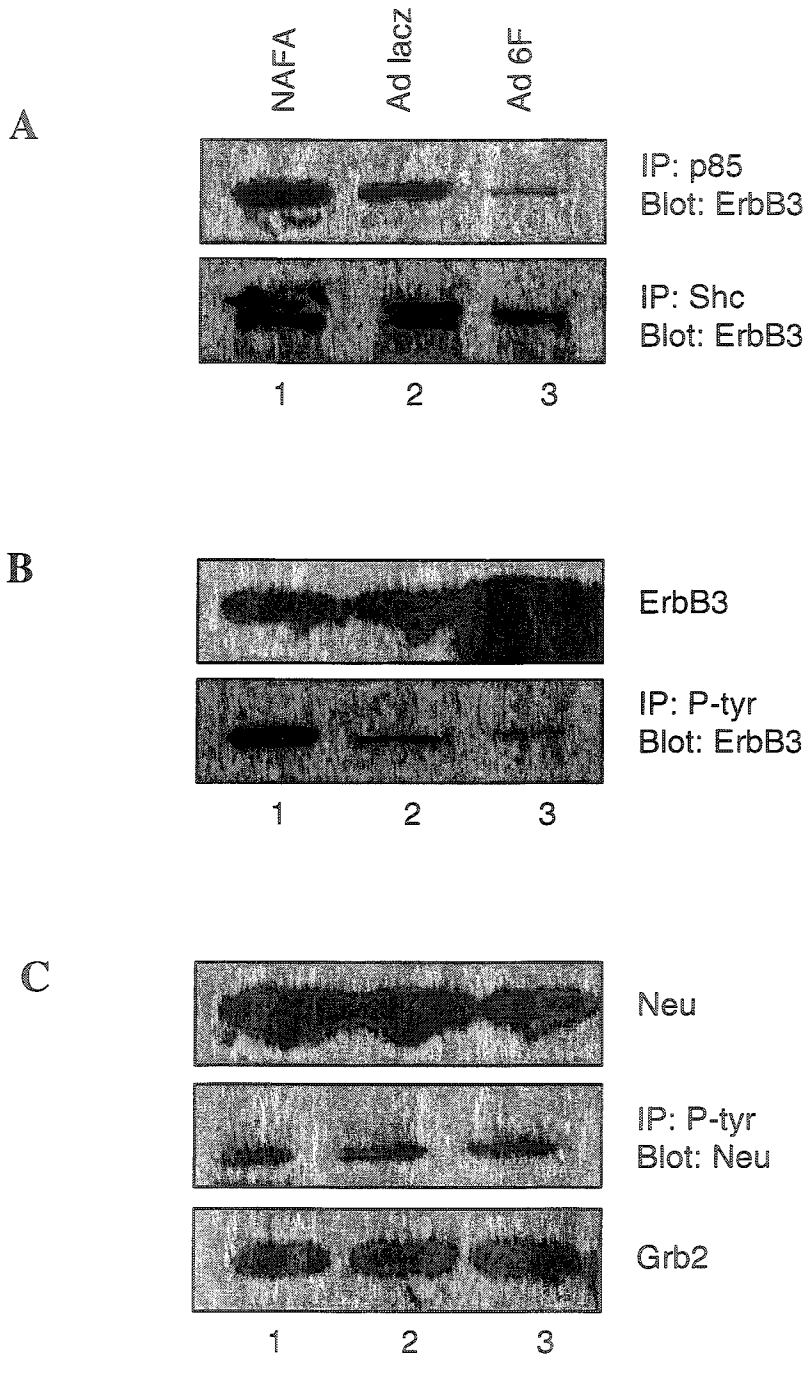

AdLacz
AdNeuKD

Figure 4.4. ErbB3-6F downregulates ErbB3 but has no effect on phosphorylation status of Neu. NAFA cells were infected with Ad6F (moi = 20) for 48 hrs. Equivalent amounts of lysates (1500 μg) from AdErbB3-6F-infected NAFA cells were immunoprecipitated with antibodies to either p85, Shc (A) or phosphor-tyrosine (B)(C). The lysates were then electrophoresed on a 4-12% gradient SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The membrane was probed with either ErbB3-specific antibodies (A)(B) or Neu-specific antibodies (C). Note the substantial decrease in the ability of ErbB3-6F mutant to bind to the p85 domain of PI-3K. Also note that the ErbB3-6F mutant still retains its ability to bind to Shc. Infection of NAFA cells with Adlacz was used as a control and note that Adlacz does not alter binding of ErbB3 to p85 or Shc. ErbB3-6F decreases ErbB3 phosphorylation but does not affect Neu.


A
NAFA
Ad lacz
Ad 6F
IP: p85
Blot: ErbB3
IP: Shc
Blot: ErbB3
1
2
3
B
ErbB3
IP: P-tyr
Blot: ErbB3
1
2
3
C
Neu
IP: P-tyr
Blot: Neu
Grb2
1
2
3

4.2.6 ErbB3-6F leads to dephosphorylation of ErbB3, S6K and 4EBP1 but has no effect on the status of Neu and Akt.

In order to determine the phosphorylation status of ErbB3 upon Ad6F treatment of NAFA cells, the protein extracts were immunoprecipitated with phospho-tyrosine antibodies and subjected to immunoblot using ErbB3-specific antibodies. Figure 4.4B shows that Ad6F significantly decreases the phosphorylation status of ErbB3. It is difficult to determine whether Ad6F has an effect of ErbB3 protein levels since the C-17 antibody used against ErbB3 cannot distinguish between wild-type and mutant ErbB3. Furthermore, Ad6F does not have an effect on total Neu protein levels nor its phosphorylation state (Figure 4.4C).

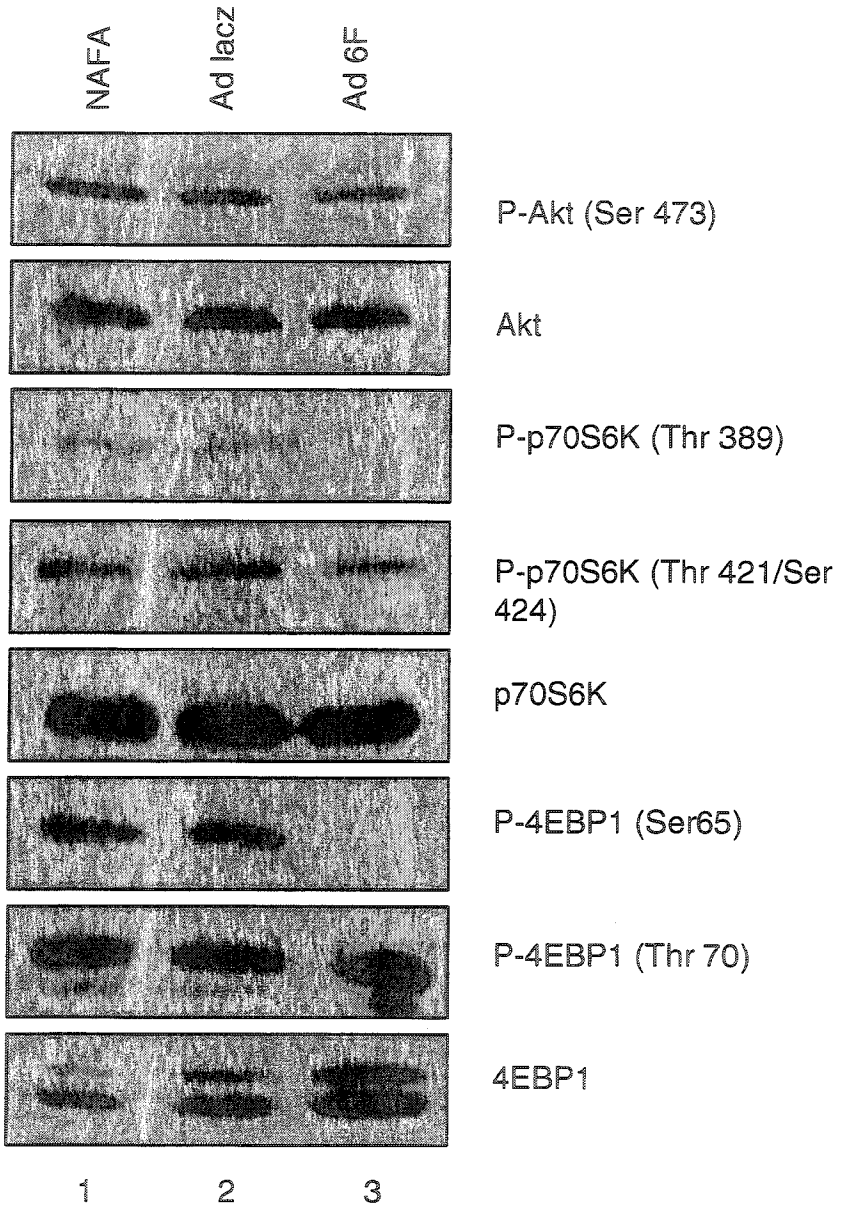
Ad6F leads to dephosphorylation of mTOR-specific Thr 389 site on S6K and mTOR-specific Ser 65 site on 4EBP1 only. Ad6F does not have an effect on phosphorylation of Thr 421/424 on S6K and Thr 70 on 4EBP1 (Figure 4.5). These results suggest that ErbB-3 is involved in the phosphorylation and activation of S6K and 4EBP1 in NAFA cells. Interestingly, inhibition of ErbB3-p85 interaction has no effect on the phosphorylation status of Akt. This finding refutes an important aspect of the hypothetical model and suggests that ErbB3 activates mTOR through mechanisms that are independent of Akt.

In addition to dephosphorylation of Ser 65 on 4EBP1, Ad6F leads to a significant increase in the levels of total 4EBP1 (Figure 4.5). This finding is interesting and suggests that ErbB36F increases the active hypophosphorylated form of 4EBP1 which would favor a decrease in protein translation.

4.2.7 ErbB3-6F decreases Neu-mediated transformation in Rat-1 fibroblasts

We determined whether ErbB3 provides cell survival signals that are necessary during Neu-mediated transformation by specifically coupling to the p85 subunit and subsequently activating PI-3K. In order to do so, focus forming assays were performed

Figure 4.5. ErbB3-6F leads to dephosphorylation of S6K and increases the amounts of active hypophosphorylated 4EBP1. Western immunoblot showing that the ErbB3-6F mutant leads to dephosphorylation S6K and 4EBP1. NAFA cells were infected with AdErbB3-6F and equivalent amounts of total cell lysates were electrophoresed on a 4-12% gradient SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. The membrane was probed with either phospho-specific Akt (Ser 473), phosphor-S6K (Thr 421/424 and Thr389), phospho-4EBP1-specific (Ser65), S6K-specific or 4EBP1-specific rabbit polyclonal antibodies. Note that the ErbB3-6F mutant does not affect Akt phosphorylation status, but does induce specific dephosphorylation of mTOR-specific Thr 389 site on S6K and Ser 65 site on 4EBP1. Ad6F does not have an effect on phosphorylation of Thr 421/424 on S6K and Thr 70 on 4EBP1. Of special note is the significant increase in total 4EBP1 protein levels upon treatment of NAFA cells with AdErbB3-6F.


NAFA
Ad lacz
Ad 6F
P-Akt (Ser 473)
Akt
P-p70S6K (Thr 389)
P-p70S6K (Thr 421/Ser 424)
p70S6K
P-4EBP1 (Ser65)
P-4EBP1 (Thr 70)
4EBP1
1
2
3

in order to determine whether inhibition of the ErbB3-p85 interaction would have an affect on Neu-mediated transformation of Rat-1 fibroblasts. cDNAs for wild type Neu (NeuN), point mutant (NT), deletion mutant (8142), wild type ErbB3 (B3) and mutant ErbB3 (6F) were used in the assay which is summarized in Figure 4.6. Coexpression of NeuNT and with wild-type ErbB3 resulted in approximately 25% increase in foci formation. Furthermore, coexpression of Neu8142 with ErbB3 resulted in a 2 fold increase in the number of foci. Co-expression of wild-type Neu with ErbB3, on the other hand, does not significantly affect transformation. Consistent with previous findings, these results suggest that wild-type ErbB3 synergizes with altered Neu receptors during Neu-mediated transformation (Siegel et al., 1999). Interestingly, coexpression of Neu8142 with the ErbB3-6F mutant decreases the number of foci formation by approximately 2 fold. These results very strongly support hypothetical model which suggests that the interaction of ErbB3 and PI-3K play an important role during Neu-mediated transformation.

4.2.8 ErbB3-6F induces apoptosis in NAFA cells prevents growth of NAFA cells *in vivo*

To test whether ErbB3 is providing antiapoptotic signals by binding to p85 subunit of PI-3K, we determined whether the ErbB3-6F mutant could induce apoptosis in activated Neu-induced tumors. NAFA cells were infected with the Ad6F virus for 48 hours. The cells were then subjected to TUNEL assay. The results in Figure 4.7 clearly demonstrate that Ad6F induces apoptosis of NAFA cells. The nuclei of the cells treated with Ad6F are stained dark brown which is indicative of apoptotic cell death. Cells undergoing apoptosis were not detected in NAFA cells treated with Adlacz suggesting that the induction of apoptosis was not a viral effect but rather due to the presence of ErbB3-6F mutant alone. The last panel is a higher magnification showing a representative cell undergoing apoptosis with its nucleus stained dark brown. These results strongly suggest that ErbB-3 is providing anti-apoptotic signals by coupling to the p85 domain of PI-3K in Neu-induced tumors.

The promising results illustrating the ability of ErbB3-6F to inhibit Neu-induced transformation of Rat-1 fibroblasts led to the testing of this ErbB3 mutant for any anti-

Figure 4.6. ErbB3-6F mutant decreases Neu-mediated transformation of Rat-1 fibroblasts. Bar graph illustrating results of focus forming assays using activated Neu and ErbB3-6F mutant. cDNAs for wild type Neu (NeuN), point mutant (NT), deletion mutant (8142), wild type ErbB3 (B3) and mutant ErbB3 (6F) were cloned into pJ4Ω construct containing the MoMULV LTR promoter. The constructs were cotransfected using lipofectamine and the transforming ability was determined by focus forming assays using rat-1 fibroblasts. Note that while B3 increases Neu-mediated foci formation by approximately 50 %, 6F mutant leads to a decrease in Neu-mediated foci formation suggesting the importance of the interaction of ErbB3 and PI3'K during Neu-mediated transformation. Error bars represent standard deviation.

ErbB3-6F Mutant Decreases Neu-induced Transformation in Rat-1 Fibroblasts

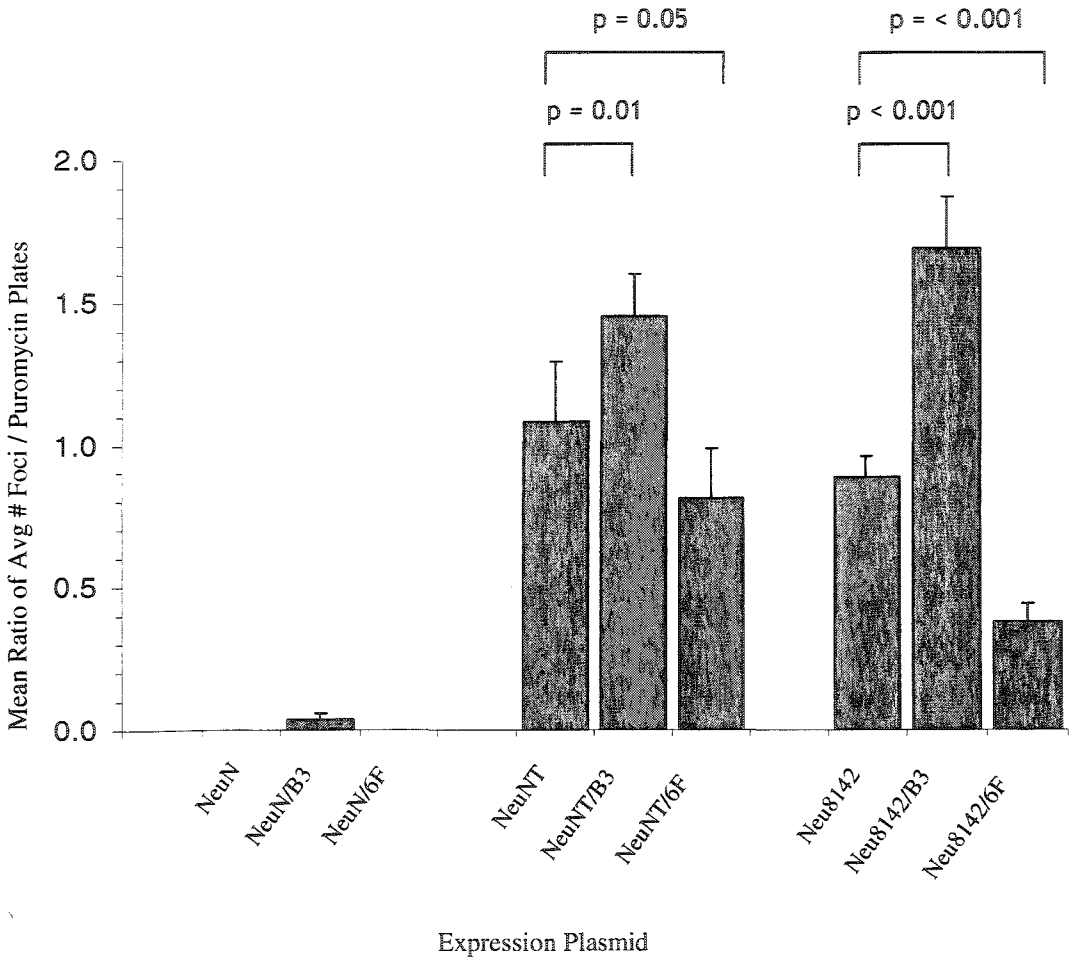
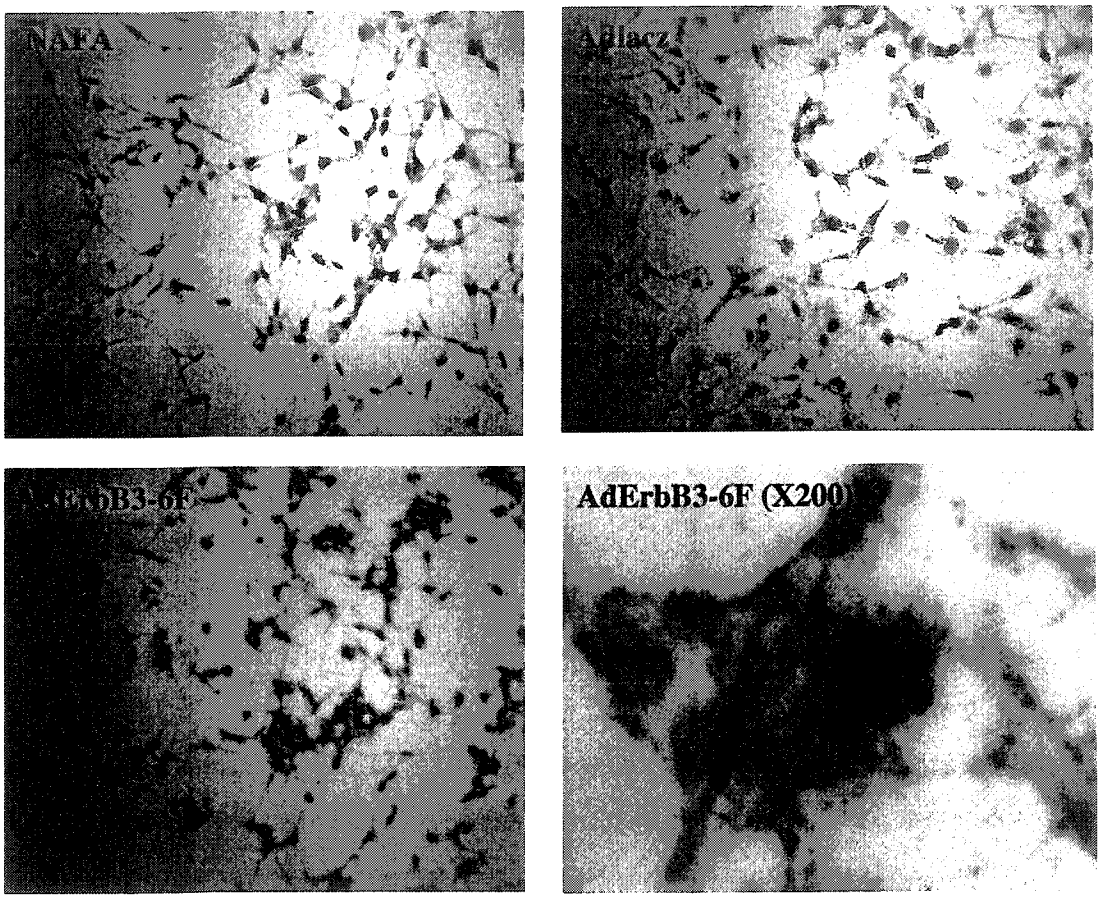


Figure 4.7. Mutant ErbB3-6F induces apoptotic cell death. TUNEL analysis showing induction of apoptosis in NAFA cells after 48 hr treatment with AdErbB3-6F. NAFA cell lines were infected with either control Adlacz virus or AdErbB3-6F followed by TUNEL assay. AdErbB3-5F leads to apoptotic cell death whereas cells left untreated or infected with Adlacz do not show signs of apoptotic cell death. Cells undergoing apoptosis have their nucleus stained dark brown. The last panel is a higher magnification (×200) showing a representative cell with its nucleus stained brown (apoptotic) or blue/green (normal).


NAFA
Adlacz
AdErbB3-6F
AdErbB3-6F (X200)

tumor activity *in vivo*. To determine whether ErbB3-6F inhibits or decreases the rate of tumor growth of NAFA cells *in vivo*, a transplanted mouse clinical trial was performed.

NAFA cells were infected with either Ad6F, Adlacz or mock for 48 hours after which the cells were transplanted subcutaneously into Fvb female mice. Mice were randomized to receive either Ad6F,Adlacz or mock infected NAFA cells. Each of the three groups consisted of 5 mice each. The tumor volumes in all three groups were measured weekly using calipers. The mice were euthanized at 5 weeks post-transplant since the tumors had reached ethical endpoint. Figure 4.8 depicts the results of this trial. The control groups (NAFA, Adlacz) show a linear increase in tumor volume between 1 to 3 weeks post-transplant. Interestingly however, the Ad6F group did not start to develop measurable tumors until week 2. Although tumor growth was slow between week 2 and 3, the growth dramatically increased after week 3 reaching 0.5 cm^3 by week 4. The tumor volume of the Ad6F group was very comparable to that of the NAFA group at weeks 4 and 5. The transient nature of ErbB3-6F expression in these cells may account for the differential pattern of tumor growth in mice transplanted with ErbB3-6F-infected NAFA cells. The results of this trial suggest that the ErbB3-6F mutant delays the onset of Neu-induced tumors.

The ability of the ErbB3-6F mutant to delay tumor onset and growth compounded with the fact that this mutant leads to apoptotic cell death, led to determining whether Ad6F is capable of regressing tumors *in vivo*. A randomized mouse placebo controlled trial was performed in order to test the anti-tumor efficacy of Ad6F. Six groups of five mice (n=5) each were used. All mice were transplanted with untreated NAFA cells (10^7 cells) in the right flank. The groups received intratumoral injections of either PBS (NAFA), Adlacz or Ad6F. The first group received Ad6F after 1 week of tumor transplantation. The second group received intratumoral injections of Ad6F after 2 weeks and so on. The therapeutic benefit obtained by injecting Ad6F into Neu-induced tumors *in vivo* are summarized in Figure 4.9. One week after NAFA transplantation, the tumors grew to a size that was difficult to measure using calipers, however, it could be detected by palpation of the tumor transplanted site. Injections of Ad6F after 1 week post-transplantation resulted in complete tumor regression. However, when the tumors were allowed to grow either 2 or 3 weeks before initiation of intratumoral injections of Ad6F,

Figure 4.8 Mutant ErbB3-6F prevents the growth of NAFA cells *in vivo*. Graph showing that ErbB3-6F inhibits tumor progression. NAFA cells were infected with either mock, Adlacz or Ad6F (moi=5) for 48 hrs. Cells were then removed from tissue culture plate, washed 3X in PBS and transplanted into the right flanks of Fvb/n mice. Each of the three groups consisted of 5 mice. The tumor volumes in all three groups (n=5) were measured weekly using calipers. Note that Ad6F delays tumor onset although tumor volume starts to increase after 2 weeks. Also note that Adlacz behaves similarly to the control vehicle group.

ErbB3-6F Mutant Prevents Neu-induced Tumor Growth

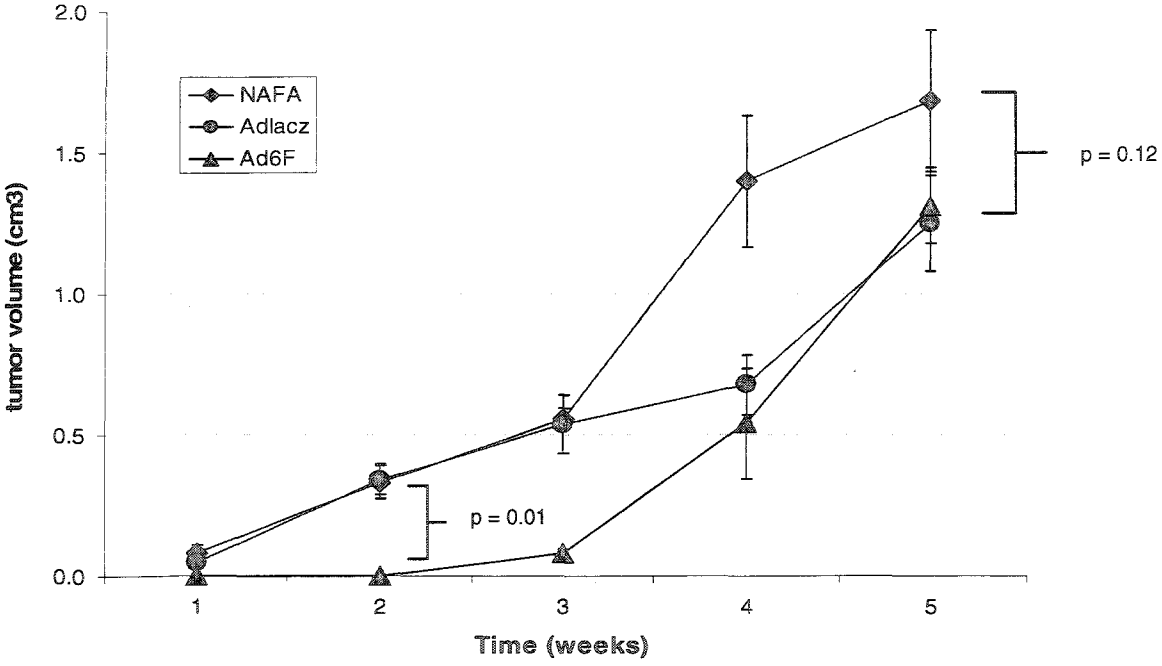
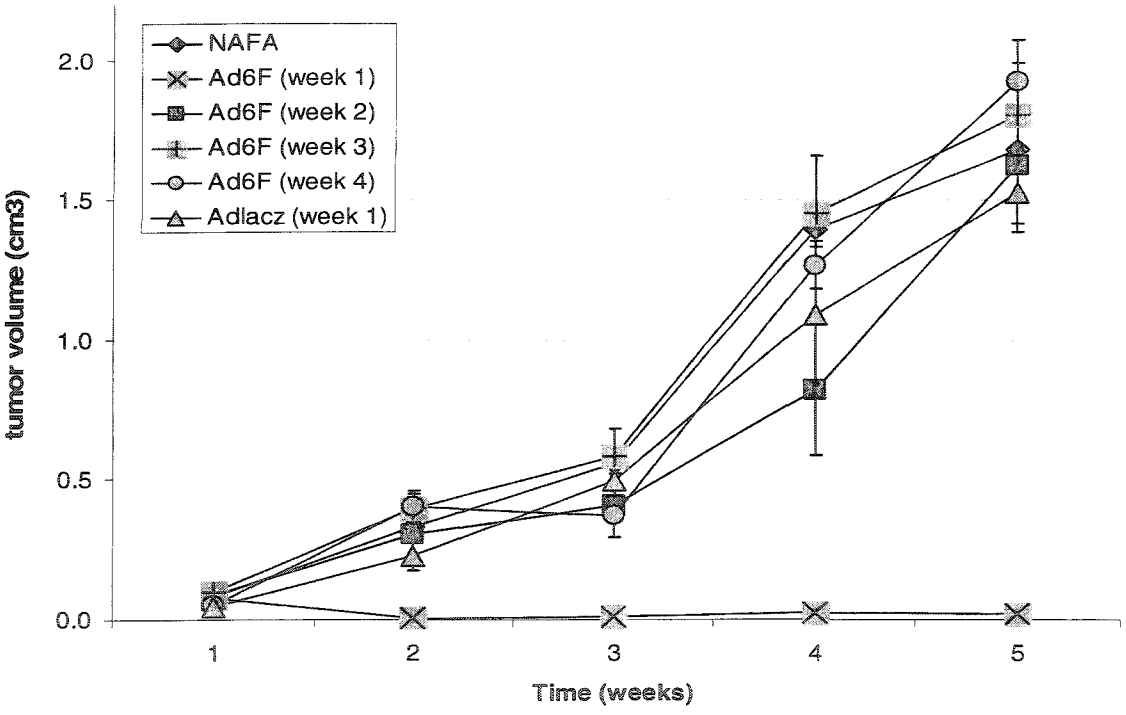


Figure 4.9 Mutant ErbB3-6F facilitates Neu-mediated tumor regression. Graph showing that ErbB3-6F mutant facilitates Neu-mediated tumor regression. Each of the 6 groups consisted of 5 mice (n=5). All mice were transplanted with untreated NAFA cells (10^7 cells) in the right flank. The groups received intratumoral injections of either Adlacz or Ad6F at various times points after palpable tumor onset. At week one, tumors were palpable and measured approximately 0.2 cm^3. Intratumoral injections of AdErbB3-6F after week 1 led to complete regression of the palpable tumor. Note that intratumoral injections of AdErbB3-6F beginning at week2,3 and 4 post-transplant had no effect on tumor regression.

ErbB3-6F Mutant Facilites Neu-induced
Tumor Regresssion



the tumors fail to regress. In summary, the tumor regresses and remains regressed if treatment is given at the earliest time-point (1 week). Treatment after 1 week does not affect tumor size. The results of this mouse randomized controlled trial suggest that although the effect of Ad6F on tumor growth is not robust, these preliminary observations suggest that the ErbB3-6F mutant has anti-tumor properties that are worth investigating further.

4.3 DISCUSSION

The results in this chapter illustrate that constitutive tyrosine phosphorylation of ErbB3 is dependent on the activity of overexpressed Neu. More importantly, the activity of PI-3K and mTOR targets (S6K and 4EBP1) are fully dependent on the activity of Neu and the ability of phospho-ErbB3 to recruit the p85 regulatory unit of PI-3K. Finally, inhibition of ErbB3/p85 interaction is apoptotic and decreases the proliferation of Neu-induced tumors in cell culture and *in vivo*. These results provide possibly the first direct evidence for a significant role for ErbB3 in Neu-induced tumors, and strongly suggests that Neu requires ErbB3-dependent activation of PI-3K/mTOR cell survival signaling pathway for its full oncogenic potential.

Evidence suggesting that Neu/ErbB3 co-receptor activity is important *in vivo* comes from studies using MMTV-Neu Deletion mutant transgenic mouse tumor model showing that endogenous expression and phosphorylation of ErbB3, but not ErbB1 or ErbB4 was increased in these tumors (Siegel et al., 1999). In addition to this, the study also showed that co-expression of Neu and ErbB3 had a synergistic effect on foci formation in Rat-1 fibroblasts (Siegel et al., 1999). Further evidence for the importance of Neu/ErbB3 co-receptor activity comes from studies using ErbB2-overexpressing human breast tumor cell lines. In these studies, loss of functional ErbB3 in SKBR3, MB361 and BT474 was strongly antiproliferative. Interestingly, each of these cell lines also express ErbB1 and BT474 expresses ErbB4. However, neither ErbB1 nor ErbB4 could drive cell proliferation by replacing ErbB3 as the heterodimerization partner for ErbB2 (Holbro et al., 2003). These studies support the notion that ErbB3 is the biologically relevant partner for overexpressed Neu.

As mentioned earlier, activation of kinase dead ErbB-3 during Neu-mediated mammary tumorigenesis most likely results from transmodulation by Neu. In order to explore the contribution of Neu/Ras/MAPK proliferative pathway in ErbB3/PI-3K/mTOR regulation, NeuKD was used to inhibit the function of NeuNT by formation of inactive homodimers. Rat-1 fibroblasts containing inactive NeuNT/NeuKD dimers lost complete transforming ability when compared to rat-1 fibroblasts containing NeuNT homodimers. Furthermore, NAFA cells infected with NeuKD (therefore containing NeuNT/NeuKD inactive dimers) showed a dramatic decrease in the signaling potential of the ErbB3/PI-3K/mTOR pathways. This interesting finding not only supports the hypothesis but also supports other studies that have shown that a major consequence of targeting overexpressed ErbB2 is a substantial decrease in PI-3K activity (Basso et al., 2002; Lane et al., 2000; Neve et al., 2000). Further evidence supporting these results come from studies showing that in ErbB2 overexpressing breast tumor cell lines, there is a correlation between ErbB3 expression levels and sensitivity to ErbB2-directed inhibitors (Holbro et al., 2003; Lane et al., 2001; Munster et al., 2002). For example, the MKN7 tumor cell line, which has high levels of ErbB2 but lacks ErbB3, is insensitive to ErbB2-directed inhibitors such as 4D5 and 17-AAG (Lane et al., 2001; Munster et al., 2002). Further evidence comes from studies showing that intracellular retention of ErbB2 leads to a dramatic dephosphorylation of ErbB3 along with a decrease in its ability to associate with PI-3K and a subsequently inhibit proliferation of breast cancer cells (Neve et al., 2000). Furthermore, treatment with HER2 blocking antibody, herceptin, blocks the constitutive phosphorylation of ErbB3, disrupts the ErbB3/p85 association and inhibits cell proliferation in BT-474 cells (Yakes et al., 2002). Although the above mentioned studies only elude to the fact that the ErbB3/p85 interaction may be important for proliferation, they do not directly assess the biological significance of this interaction. These results suggest that mitogenic signaling pathways originating from activated Neu result in activation of ErbB3/PI-3K/mTOR pathways.

A much more novel and interesting finding was that infection of NAFA cells with AdNeuKD led to a decrease in ErbB3 protein levels as well as decrease in its phosphorylation state. NeuKD also led to dephosphorylation of S6K and 4EBP1. Strikingly, inhibition of Neu had a dramatic impact on the phosphorylation of the

Thr421/424 site on S6K. This supports studies that have shown that S6K may be phosphorylated at this site by mTOR-insensitive pathways such as Ras/MAPK and that MAPK signal-integrating kinases like Mnk1/2 can phosphorylate 4EBP1 independent of mTOR. One explanation for this is that inhibiting the ability of Neu to transphosphorylate and activate ErbB3 prevents ErbB3 from binding to PI-3K and activating mTOR, 4EBP1 and S6K.

The fact that NeuKD induced apoptosis in NAFA cells in addition to downregulation of ErbB3, suggests that ErbB3 in conjunction with ErbB2 may be activating the antiapoptotic pathway. Indeed studies have shown that intratumoral injections of an adenovirus expressing NeuKD has a therapeutic effect on ErbB2-induced tumor growth by inducing apoptosis (Palmer et al., 2002). However, the mechanism of NeuKD-induced apoptosis has yet to be addressed. Biochemical results obtained in this chapter from NeuKD-infected NAFA cells suggests that one mechanism by which NeuKD may be facilitating ErbB2-induced tumor regression and apoptosis may be through dephosphorylation of ErbB3 as well as decrease in its protein levels which subsequently results in downregulation of the essential cell survival signals provided by PI-3K/mTOR pathways. Therefore, targeting ErbB2-driven tumors with agents that disrupt formation of an active ErbB2/ErbB3 heterodimer may be useful. In this respect, it was recently shown that mAb 2C4, which blocks ErbB2's ability to heterodimerize with ErbB3, efficiently inhibits the growth of BT474 tumor cells in a xenograft model (Agus et al., 2002).

Among the known intracellular effects of the Neu/ErbB3 coreceptor are Ras/MAPK and PI-3K signaling. Several studies have suggested that PI-3K is the preferred partner for ErbB3 since it has six docking sites for p85 adaptor subunit of PI-3K compared to one Shc-binding site (NPXY1325)(Vijapurkar et al., 1998). Complexes of ErbB2, ErbB3 and p85 have been reported in breast carcinoma cell lines (Ram and Ethier, 1996). Some studies suggest that the constitutive ErbB3/p85 complexes are present due to ligand independent expression of ErbB2 while others show that these complexes are present due to the presence of HRG/ErbB3 autocrine loop in a number of ErbB3 expressing breast cancer cells (Alimandi et al., 1995; Kim et al., 1994; Kraus et

al., 1993; Ram and Ethier, 1996). However, very little is known about the significance of the ErbB2/ErbB3/p85 complex.

Biochemical analysis of the Ad6F-infected NAFA cells clearly illustrate that substitution of the six tyrosine residues with phenylalanine did not alter the conformation of the C-terminal domain of ErbB3 such that the Shc binding site was less accessible. This was important in order to confirm that any proliferative or signaling changes observed by the introduction of ErbB-6F is strictly due to the inability of ErbB3 to recruit p85. The observation that NAFA cells lacking ErbB3/p85 interaction still maintain the same high levels of phosphorylated Neu as do control NAFA cells after 48 hrs yet show a dramatic dephosphorylation of S6K (Thr 389) and 4EBP1 (Ser65) highlights the essential role of ErbB3 in recruiting PI-3K and activating mTOR-specific targets. The most striking result is the site-specific dephosphorylation of S6K and 4EBP1 by ErbB3-6F. Phosphorylation of Thr421/Ser424 sites on S6K remain unaffected in cells lacking ErbB3/p85 interaction, however S6K is completely dephosphorylated at the Thr 389 site. This strongly implicates that the PI-3K-induced phosphorylation of the rapamycin-sensitive site on S6K is completely dependent on the ability of phospho ErbB3 to interact with p85. Support for this finding come from studies with the insulin receptor substrate-1 (IRS-1). Engagement of insulin receptor by insulin leads to recruitment of the IRS-1 protein, which subsequently recruits PI-3K. Mutations within the IRS-1 that blocks the recruitment of p85 also blocks activation of S6K (Myers, Jr. et al., 1994; Myers, Jr. et al., 1996). Furthermore, treatment of cells with a PI-3K inhibitor wortmannin, blocks the insulin signal to 4EBP (Ser 65, Thr 70) and S6K (Thr 389) but does not affect Thr 421/Ser424 (Gingras et al., 2001a; Grammer et al., 1996). Studies have not clearly demonstrated the significance of Thr421/424 sites on S6K. Although it has been suggested that this site is important for S6K function, most studies agree that the Thr 389 site most parallels kinase function and leads to phosphorylation of the S6 protein on the small ribosomal unit and subsequent initiation of translation (Gingras et al., 2001b; Pearson et al., 1995). Although the Ser 65 site on 4EBP1 is regulated my mTOR, our studies show that this phosphorylation is fully dependent on the recruitment of PI-3K by ErbB3 in this particular tumor cell line. Previous studies have implicated that the phosphorylation sites on S6K and 4EBP1 are very tightly regulated. The fact that NAFA

cells lacking ErbB3/p85 interaction still contain phosphorylated Neu suggests that the MAPK signaling pathway originating from Neu is still active. This may be one explanation as to why there are no changes in the phosphorylation status of the Thr421/Ser424 site on S6K. Indeed studies have shown that Ras/MAPK pathway can phosphorylate S6K at Thr421/424 which is independent of mTOR function (Lehman et al., 2003). As for the phosphorylation sites on 4EBP1, studies have provided evidence showing that 4EBP1 is phosphorylated at Thr37, Thr46, Thr70, Ser65 sites in a sequential fashion until the final phosphorylation of Ser65 which is required for 4EBP1 to dissociate from eIF4E. The striking result that ErbB3 regulates Ser 65 phosphorylation strongly raises the possibility that in Neu-induced tumors, ErbB3 plays a key role in maintaining 4EBP1 in its inactive, eIF4E-unbound state thereby allowing uncontrolled translation of proteins predominantly regulated by mTOR.

Another very striking result is the fact that NAFA cells lacking ErbB3/p85 interaction show an increase in total 4EBP1 protein levels compared to controls. Therefore, one mechanism by which ErbB3-6F may downregulate the PI-3K and mTOR pathways may be by increasing the total levels of hypophosphorylated 4EBP1. This would lead to an increase in eIF4E-4EBP1 complexes and a further suppression of mTOR-regulated proteins. Whether the regulation of 4EBP1 by ErbB3 is at the mRNA level as well remains to be determined. Nonetheless, these results strongly implicate that one potential signaling role of ErbB3 in Neu-induced tumors may be to sustain 4EBP1 in its inactive hyperphosphorylated state thereby increasing translation of proteins involved in cell growth and proliferation.

Interestingly, results in the previous chapter suggest that mTOR / 4EBP1 pathway itself may be involved in the regulation of ErbB3. Therefore, another potential reason for ErbB3 to activate the mTOR/4EBP1 pathway may be to activate the pathway that is required to sustain high levels of endogenous ErbB3 protein. There is a possibility that under normal biological conditions, the actions of ErbB3 restrict the extent of mTOR activity, which in turn restricts the stability of ErbB3 itself. However, in Neu-overexpressing tumor cells, continuous phosphorylation and activation of ErbB3 leads to uncontrolled activity of mTOR which ultimately leads to an increase in translational efficiency of target proteins such as ErbB3.

The ability of the Neu/ErbB3-6F co-receptor to decrease the number of foci of Rat-1 fibroblasts expressing the point and deletion mutants of Neu highly suggests that ErbB3-induced recruitment of p85 is required for Neu-induced proliferation. In this regard studies have shown that interaction of ErbB3 with PI-3K is correlated with malignant phenotypes of adenocarcinomas (Kobayashi et al., 2003). In particular, this study demonstrated that ErbB3 is constitutively phosphorylated and that this activation of ErbB3 results in activation of PI-3K in signet-ring cell carcinoma cells. Furthermore, expression of the constitutively active ErbB2/ErbB3 chimeric receptor revealed that PI-3K signaling triggered by ErbB3 phosphorylation was essential for phenotypes such as loss of cell-cell interaction (Kobayashi et al., 2003).

ErbB3-6F was not as potent in inhibiting transforming ability of neu harbouring the point mutation in the transmembrane domain as it was for the deletion mutant. Although both the point and deletion mutants of Neu lead to constitutive dimerization and activation, the deletion mutant is weaker in its ability to transform Rat-1 fibroblasts (Siegel and Muller, 1996) for reasons not understood. The potent nature of NeuNT may be one explanation as to differences in proliferative block by ErbB3-6F. Nonetheless, these results highlight the importance of PI-3K activation in these tumors.

Studies have investigated the relative contributions of the MAPK and PI-3K pathways to the mitogenic and transforming potential of the ErbB2/ErbB3 activated coreceptor and have shown that the PI-3K anti-apoptotic pathway clearly predominates in the induction of cellular transformation (Vijapurkar et al., 2003). In support of this finding, studies have shown that breast cancer cell lines with ErbB2 overexpression possess an increased growth requirement from PI-3K and S6K (Hermanto et al., 2001) and are very sensitive to inhibitors of this pathway. The underlying mechanism of the increased sensitivity to PI-3K and S6K has not been addressed. One possibility may be that this phenomenon may be directly related to the phosphorylation status of ErbB2. Low ErbB2-expressing breast cancer cell lines were not sensitive to PI-3K inhibition, however, forced expression of ErbB2 in the same cell lines led to a dramatic increase in sensitivity to PI-3K inhibition (Hermanto et al., 2001).

Another possibility is that the ErbB3-mediated amplification of PI-3K signaling may result in downregulation of other pathway(s) that have a function equivalent to the

PI-3K pathway and that are otherwise active and important in facilitating tumor growth. As a consequence, ErbB-2 overexpressing cell develop a greater reliance on PI-3K signaling in anchorage-independent growth and therefore have increased sensitivity to inhibition of PI-3K signaling. The results described in this chapter along with the above findings may suggest that ErbB3 plays a key role in the increase in ErbB2-associated PI3K activity and may indict ErbB3 overexpression in the genesis and maintenance of Neu-induced tumors.

The results in this chapter strongly suggest that as a potential regulator of cell growth and proliferation, ErbB3 mediates a PI-3K-dependent mitogenic signaling pathway essential for the growth of tumors harboring amplification of the Neu oncogene. This renders ErbB3 and its downstream signal transducers, such as PI-3K and mTOR as potential targets for the treatment of breast tumors. To explore this possibility, we examined the ability of an adenovirus expressing ErbB3-6F to inhibit the growth of a Neu-induced tumor cell line (NAFA) *in vivo*. Interestingly, mice transplanted with AdErbB3-6F-infected NAFA cells show a delay in tumor onset by approximately 2 weeks. The tumor then starts to grow after 2 weeks and reaches tumor volumes comparable to the control groups. The explanation for this may be simply due to the transient nature of adenoviral infection. Since the ErbB3-6F mutant is no longer being overexpressed at 2 weeks, we see an increase in tumor volume from perhaps those cells that have escaped the mechanism of tumor inhibition. AdErbB3-6F also showed an effect on tumor regression only if the virus was intratumorally introduced when the tumor was palpable (1 week post-transplant). The mechanisms of tumor regression in these mice were not studied. However, since ErbB3-6F leads to apoptotic cell death of NAFA cells, we can speculate that tumor regression may be a result of apoptosis of the cells. Another possibility may be that AdErbB3-6F provides tumor antigens to dendritic cells through apoptotic bodies and stimulates an antitumor immune response. In support, Ronchetti et al. have shown that injection of apoptotic tumor cells elicits an antitumor immune response (Ronchetti et al., 1999). The inability of AdErbB3-6F to regress tumors that grew beyond 1 week may be simply due to the inability to reach therapeutic efficacy. Perhaps the therapeutic effect of AdErbB3-6F could be enhanced by the addition of a dominant negative ErbB member such as NeuKD. The ErbB3-6F tumor

studies further support the strong biological role of ErbB3 during ErbB2-mediated transformation. The fact that ErbB3-6F mutant did not lose its ability of associate with the Shc binding site (Y1325) certainly highlights the importance of the interaction of ErbB3 with PI-3K and strongly suggests that ErbB3 may be providing survival signals by recruiting the essential antiapoptotic pathway during Neu-mediated tumorigenesis. In support of these findings, studies done by Holbro et al (2003) have also highlighted the importance of ErbB2/ErbB3 heterodimer-dependent PI-3K signaling in breast cancer cell proliferation. They demonstrated that ErbB3 is an essential partner in the transformation process and that lack of ErbB2 or ErbB3 has similar effects on cell proliferation and cell cycle regulators (Holbro et al., 2003). Another study demonstrated that amplification of PIK3CA, which encodes the catalytic subunit (p110α) of PI3K, is amplified in human ovarian tumors (Shayesteh et al., 1999) and cervical cancers (Ma et al., 2000). Interestingly, many of the ovarian cancer cells overexpress ErbB2 but lack ErbB3 expression suggesting that PI3K may substitute for elevated ErbB3 protein in these tumors.

Results from the *in vivo* randomized controlled mouse trial suggest that ErbB3 may be a potential therapeutic target for those tumors that contain ErbB3-driven amplification of PI-3K/mTOR pathways. In support of this, studies have shown that tumors containing ErbB2/ErbB3/p85 complexes are the most potent signaling complex in terms of growth and transformation and can lead to enhanced chemotherapeutic drug resistance (Alimandi et al., 1995; Alroy and Yarden, 1997; Chen et al., 2000; Ram and Ethier, 1996). Although the reason for the high potency is not well understood, it may be that this potency is related to the ability of ErbB3 to recruit p85 and activate mTOR and other potentially novel downstream signaling molecules.

Recent studies have focused on determining a more detailed mechanism by which Neu leads to induction of mammary tumors. Identification of the precise roles played by mitogenic and anti-apoptotic signal transduction pathways in Neu-mediated tumors will lead to both a better understanding of the mechanisms underlying the biological behaviour of Neu-induced tumors and the potential development of effective strategies to treat those tumors. Demonstration of ErbB3's essential role in cell survival by coupling to p85 and activating S6K and 4EBP1, provides a model that could explain why

preventing the activity of ErbB3 as well as ErbB2/ErbB3 heterodimers may have a therapeutic effect in tumors that overexpress ErbB2.

CHAPTER 5

Conclusions and Future Directions

5.1 CONCLUSIONS

The progression of mammary epithelial cells to the malignant phenotype involves cumulative genetic changes that alter growth signal transduction pathways governing cell proliferation. Proto-oncogenes and tumor suppressor genes regulate normal cellular growth and are frequently altered in breast cancer. Of relevance to this thesis is the observation that activating RTKs are amplified in the genesis of human breast cancer. In this regard, overexpression of wild-type ErbB2, generally attributable to gene amplification, occurs in 25-30% of human breast cancer and correlates with short time to relapse and lower overall survival (King et al., 1985; Slamon et al., 1987; Slamon et al., 1989; Yokota et al., 1986). Previous studies confirm the oncogenic potential of the Neu (the rat homologue of ErbB2) receptor through the generation of transgenic mice expressing wild type Neu in the mammary gland (Guy et al., 1992b). Furthermore, Siegel et al. had shown that activating mutations within the extracellular domain of Neu were present in 65% of the mammary tumors arising in the MMTV/wt Neu strain (Siegel et al., 1994). Moreover, these altered Neu receptors had the capacity to induce tumors when expressed in the mammary gland of transgenic mice (Siegel and Muller, 1996) suggesting that the activating mutations in Neu play a causal role in the transformation process.

Siegel et al also characterized these activated Neu-induced tumors which revealed a potential collaborating event with another EGFR member, namely ErbB3, during Neu-mediated tumorigenesis. Specifically, endogenous ErbB3 protein levels were found to be uniformly upregulated by 10-20 fold in mammary tumors overexpressing Neu (Siegel et al., 1999). The presence of equivalence levels of ErbB3 transcript in normal versus tumor tissues suggests that this increase is a result of either increase translation or stability of the ErbB3 protein. However, the mechanism of ErbB3 translational and/or post-translational regulation needs to be elucidated.

Previous studies have also demonstrated that the overexpressed endogenous ErbB3 observed in Neu-induced tumors was constitutively tyrosine phosphorylated which suggests an active signaling role for this receptor during Neu-mediated transformation (Siegel et al., 1999). ErbB3 itself however, has impaired tyrosine kinase activity (Guy et al., 1994b) and needs a dimerization partner to become phosphorylated and acquire signaling potential (Kim et al., 1998). Since ErbB2 is overexpressed in these tumors, it is the most likely candidate to transphosphorylate and activate ErbB3. The above observations compliment published results demonstrating elevated levels of ErbB2 and ErbB3 in human breast cancers (Naidu et al., 1998; Siegel et al., 1999).

Clinical studies have attempted to report the significance of ErbB3 expression in breast cancer. Expression of ErbB3 was investigated in 104 primary breast carcinomas. A high percentage (67%) of ErbB2 positive DCIS was strongly positive for ErbB3. Furthermore, a significant relationship between ErbB3 and histological grade was observed in these tumors. Many studies agree that there is a tendency for an observed increase in tumor size and a decrease in survival prognosis in patients that overexpress ErbB3 (Bacus et al., 1996; Gasparini et al., 1994; Knowlden et al., 1998; Srinivasan et al., 2000; Travis et al., 1996; Vogt et al., 1998). Indeed earlier studies performed with other types of tumors attribute clinical significance to co-expression of ErbB receptors. For example, co-expression of ErbB2 with ErbB3 critically improved the predicting power of patient outcome in oral squamous cell cancer (Xia et al., 1999). Perhaps future studies on breast tumors may reveal similar relationships. Taken together, these clinical findings suggest that overexpression of ErbB3 could play an important role during tumor progression. However, the significance of ErbB3 overexpression in Neu-mediated tumorigenesis remains unknown. .

An important question to be addressed is whether ErbB3 phosphorylation is a mere consequence of ErbB2 activation or whether it is necessary for ErbB2's full oncogenic potential. One explanation for this co-expression may be that both ErbB2 and ErbB3 recruit distinct yet complimentary signaling pathways that cooperate during mammary tumor progression. In support of this hypothesis, studies have shown that cell proliferation and cell survival are distinct yet coupled processes that are required for complete transformation (Amundadottir et al., 1996; Lee et al., 2003; Menu et al., 2004;

Xia et al., 2002). It has been previously shown that ErbB2 sends strong proliferative signals predominantly through the Ras/MAPK pathway. Perhaps the role of ErBb3 in these tumors is to provide cell survival signals by recruiting the p85 regulatory unit of PI-3K and activating mTOR and its downstream targets. Transgenic mouse models for breast cancer support this hypothesis (Amundadottir et al., 1996; Hutchinson et al., 2001; Webster et al., 1998). One example supporting the importance of PI-3K/Akt cell survival pathway during transformation derives from studies using transgenic mice overexpressing a mutant PyVmT (mT) antigen (Y315/322F) uncoupled to the PI-3K pathway (Webster et al., 1998). These mice develop apoptotic epithelial hyperplasias compared to wild-type mT antigen-expressing transgenics that develop multi-focal metastatic mammary tumors (Guy et al., 1992a). Further support for the importance of survival signals during mammary tumorigenesis comes from bigenics expressing the Y315/322 mT mutant and a constitutively activated form of Akt (Hutchinson et al., 2001). These bigenics show a dramatic acceleration of mammary tumorigenesis, compared to Y315/322 mT mice, which correlates with reduced apoptotic cell death and a significant upregulation of cyclin D1. These studies suggest that PI-3K contributes to tumor progression by providing important cell survival signals.

Studies have shown that the PI-3K-dependent activation of the mTOR/4EBP1 signaling pathway is critical for cell growth and survival (Bretland et al., 2001; Jacinto and Hall, 2003; Martin and Blenis, 2002; Raught et al., 2001; Vogt, 2001). Dysregulation of this pathway generates a favourable oncogenic environment and has been documented in a variety of transformed cells and human tumors (reviewed in (Hidalgo and Rowinsky, 2000; Mita et al., 2003b). Although mutations of mTOR itself have not been reported, mutations in the components of the mTOR-related signaling pathways have frequently been described in human malignant diseases including breast cancer (Dancey, 2002; Huang and Houghton, 2002; Vogt, 2001). Elevated levels or constitutive activation of growth factor receptors, PI-3K, Akt, S6K, 4EBP1 and cyclin D1 are found in breast tumors.

The focus of this thesis was to determine the molecular pathway (s) involved in ErbB3 regulation and the biological significance of ErbB3 during Neu-mediated tumorigenesis. The results of this thesis conclude the following and are illustrated in

Figure 5.1: (1) Both PI-3K and mTOR are involved in sustaining the high levels of endogenous ErbB3 by increasing the stability of the protein (2) A major consequence of inhibiting Neu is dephosphorylation and downregulation of ErbB3 and members of the PI-3K/mTOR pathway suggesting that ErbB3 depends on Neu for its activation. This supports the hypothesis that both the proliferative and survival pathways are coupled (3) Further support for collaboration of these two pathways comes from results demonstrating that activation of the PI-3K/mTOR pathway depends on the ability of phospho-ErbB3 to recruit and activate PI-3K (4) ErbB3 in conjunction with Neu, is involved in the activation of S6K, 4EBP1 and cyclin D1 (5) Importantly, inhibition of mTOR in a mouse tumor model delays tumor onset and progression which correlates with a decrease in ErbB3 activation and S6K/4EBP1 phosphorylation.

Taken together, these results demonstrate that the PI-3K/mTOR pathway, in part, accounts for the increase in endogenous ErbB3 protein observed in Neu-induced tumors. Moreover, Neu requires anti-apoptotic signals provided by ErbB3 for its full oncogenic potential suggesting that the Neu/ErbB3 heterodimer functions as a unit during Neu-mediated mammary tumorigenesis. Importantly, the results of the mouse randomized controlled trial provides a rationale for the use of the rapamycin analogue CCI-779 in the therapy of those breast tumors that overexpress both HER-2 and ErbB3.

5.2 FUTURE DIRECTIONS

Interesting findings in this thesis lend themselves to future studies. This section highlights future experiments to improve the understanding of the molecular mechanism of ErbB3 regulation and its role during Neu-mediated mammary tumorigenesis.

As cited in Chapter 3, NDL transgenic mice demonstrated significant response to rapamycin treatment, supporting the mechanism observed in NAFA cells in this thesis. In the future, PI-3K/mTOR-dependent regulation of ErbB3 should be tested in a wide range of breast tumor cell lines. However, despite the historical significance of long-term *in vitro* cultures (Boyd MR, 1989; Goldin et al., 1979; Monks et al., 1991), there is often significant phenotypic diversity between established cell lines and primary tumors from which they were derived (Band et al., 1990; Lukashov and Goudsmit, 1995; Sen et al.,

1995). Selection both during initial culture and long-term propagation may lead to important biological differences between different sublines (Band et al., 1990; Crepin et al., 1990; Hiorns et al., 2004; Lukashov and Goudsmit, 1995; Osborne et al., 1987). These above findings provide supportive data for evidence that cell lines do evolve in culture, thereby weakening the direct relevance of such cultures as models of human cancer. Therefore, the hypothetical model presented in Figure 1.2 must also be tested in other transplanted and transgenic mouse models.

Although the studies presented in this thesis implicate the importance of PI-3K/mTOR in the translational and/or post-translational control of ErbB3, the results do not provide sufficient evidence to understand the precise mechanism of mTOR-dependent ErbB3 regulation. Importantly, whether mTOR is involved in the regulation of ErbB3 translation needs to be determined. In a staged program of research, it should first be determined whether the polysomal representation of ErbB3 mRNA is increased in Neu-induced tumors. Next, it should be determined whether ErbB3 mRNA contains a long 5'UTR with characteristic features of a typical translationally repressed transcript. This could be confirmed by analyzing the distribution of ErbB3 mRNA after sedimentation in sucrose gradients. The effects of LY294002 and rapamycin on ErbB3 translation rate requires clarification. If inhibition of protein translation is observed then it can be concluded that PI-3K and mTOR regulate the translation as well as the stability of ErbB3. Indeed mTOR is known to regulate both the translation and stability of cyclin D1 which is PI-3K mediated (Hashemolhosseini et al., 1998; Muise-Helmericks et al., 1998; Panwalkar et al., 2004). If ErbB3 lacks polypyrimidine sequences in its 5'UTR then other mechanisms of mTOR-dependent ErbB3 translational regulation should be explored.

Recent studies support the rationale of this approach. Defatta and De Benedetti demonstrated that mTOR-regulated eIF4E exerts its full oncogenic potential by increasing the translational efficiency of oncogenes and growth-promoting transcripts normally repressed by their 5'UTR (Defatta and De Benedetti, 2003). An example of such an oncogene is src-like kinase yes. The yes kinase gene encodes a protein similar to p60src both in size and sequence (Yamamoto and Toyoshima, 1987) and has been shown to bind Neu through the same phosphorylation site utilized by c-src (Muthuswamy and Muller, 1995b). yes has also been implicated in the progression of mammary tumors by

impinging on the initial phases of mitosis (Hansen et al., 2001; Moasser et al., 1999). The yes mRNA contains polypyrimidine sequences in its 5'UTR which is a target of eIF4E (Defatta and De Benedetti, 2003). Furthermore, transformation of yes-null fibroblasts by eIF4E was significantly impaired. These findings support the possibility that eIF4E and other mTOR targets may depend on translational regulation of other oncogenes, such as overexpressed ErbB3, for their complete transforming ability.

If LY294002 and rapamycin do not inhibit protein translation then a closer look at protein degradation is required. The pulse chase analysis performed for this thesis implicates PI-3K and mTOR in the regulation of ErbB3 stability. However, future experiments should examine the reversal of LY294002 and rapamycin-induced degradation of ErbB3 with lactacystin, MG-132 and other proteosome inhibitors. One could also determine whether LY294002 and/or rapamycin lead to an increase in ubiquitinated forms of ErbB3, and whether rapamycin-resistant mTOR has any effect on the half-life of ErbB3. These studies would further solidify the notion that mTOR regulates ErbB3 stability during Neu-mediated transformation.

Although the possibility of translational and/or post-translational regulation of ErbB3 is novel, it is a phenomenon not unique to EGFR members. Initially, ErbB2 overexpression was thought to occur only through amplified copies of the gene (King et al., 1985; Yokota et al., 1986). However, studies later demonstrated that ErbB2 overexpression also involved transcriptional and post-transcriptional mechanisms (Berger et al., 1988; Guerin et al., 1988; Slamon et al., 1989). Interestingly, subsequent independent studies clearly demonstrate that ErbB2 is also regulated at the translational level (Buhring et al., 1995; Child et al., 1999a; Child et al., 1999b; Oshima et al., 1995) in human breast cancer cell lines such as BT474 and MCF-7 (Child et al., 1999a). This heightens the possibility of other EGFR members such as ErbB3 being regulated at the translational level.

The observation of Akt phosphorylation remaining unchanged when NAFA cells were treated with LY294002, rapamycin, AdNeuKD, and AdErbB36F is interesting. This most certainly refutes the hypothesis of Akt being involved in the regulation of ErbB3. Although PI-3K-dependent activation of S6K and 4EBP1 was predominantly believed to occur via Akt, recent evidence shows that PI-3K can activate S6K independently of Akt

through an alternative pathway involving PDK1 (Radimerski et al., 2002). Future experiments may determine whether a constitutively active PDK1 can prevent LY294002 and rapamycin-induced ErbB3 downregulation and proliferation. To determine whether Akt is involved in the regulation of ErbB3, NAFA cells expressing a constitutively active Akt can be treated with LY294002. If ErbB3 is downregulated, then this would strongly suggest Akt is not involved in its regulation. Alternatively, a dominant negative Akt may be used to confirm this notion.

Rapamycin had a significant effect on tumor onset and progression in both the transplanted in transgenic mouse models, confirming its antiproliferative effect on activated Neu-induced tumors. In the transplanted tumor model, tumor size at the end of study was significantly smaller in rapamycin-treated mice vs. control mice. This thesis, however, did not directly assess the mechanism by which tumor growth was inhibited throughout the course of the study. The biochemical analysis of these tumors were performed at the end of the study, and revealed no differences in phosphorylation states of Neu, ErbB3, S6K and 4EBP1, nor changes in ErbB3 or cyclin D1 protein levels (data not shown). Biochemical results may have been derived from those tumor cells which developed escape mechanisms of tumor inhibition by rapamycin. True biochemical changes may have been observed at earlier stages of the study. Future experiments should increase the sample size of mice so that a proportion can be sacrificed at various stages of the study, and the biochemical characteristics of the tumors examined. Assessing the percentage of cells arrested in G1, the phosphorylation statuses of S6K/4EBP1 and whether they correlate with ErbB3 protein levels throughout the course of the treatment would be important in directly assessing the mechanism of rapamycin-induced tumor inhibition in Neu-induced tumors.

Further experimentation is necessary to determine the extent by which ErbB3 is involved in Neu-mediated transformation. Many studies have used bigenic mice to assess the collaboration of various proteins in transforming the mammary epithelial gland (Amundadottir et al., 1996). Indeed bigenics were derived by combining mice overexpressing a transgene with mice lacking an endogenous gene (Donehower et al., 1995; Guy et al., 1994a; Hundley et al., 1997). In a similar fashion, transgenic mice overexpressing either wild-type or activated Neu can be established with a genetic

background that lacks ErbB3 expression. Given ErbB3 deficient mice die during gestation, conditional activation of ErbB3 would need to be considered.

Further research is necessary to illustrate the importance of the ErbB3-p85 interaction in providing cell survival signals by activating the PI-3K/mTOR pathway. In this regard, ErbB3-6F knock-in mice have been generated and are currently being crossed with transgenic mice overexpressing activated Neu (W.J.Muller, unpublished) The outcome of such an *in vivo* experiment may corroborate results obtained with the ErbB36F mutant (Chapter 4) .

In addition to studying the significance of the ErbB3-p85 interaction, further research should characterize the significance of the Y1325 Shc binding site located in the C-terminus of ErbB3. Since Shc has been implicated in mitogenic signaling by interacting with ErbB2 and activating the Ras/MAPK pathway (Dankort et al., 2001; Dankort et al., 1997; Saucier et al., 2004; Stevenson et al., 1999), examining the relative contributions of Shc and p85 in the induction of cellular transformation in various tumor cells would be useful. This would further detail the importance of ErbB3-p85 interaction in Neu-mediated transformation.

Although the ability of AdErbB3-6F to prevent tumor growth and facilitate tumor regression is interesting, further studies need to establish the efficacy of the adenovirus. It remains to be determined if this virus is similar in effect to rapamycin, when given systemically prior to tumor cell transplantation and prior to tumor onset in transgenic mice overexpressing Neu. The AdErbB3-6F virus may be more effective in delaying tumor onset and progression versus tumor regression. In this regard, anti-VEGF antibodies used in phase II clinical trials were shown to significantly delay the time to tumor onset and progression in kidney cancer (Yang et al., 2003). Interestingly, this antibody did not have an effect on tumor regression. Since PI-3K and mTOR are involved in the regulation of VEGF through HIF-1α (Hudson et al., 2002; Zhong et al., 2000), there is a possibility that downregulation of VEGF may be another mechanism by which rapamycin exerts its anti-proliferative effects. Similarly, based on the results of this thesis, the phase II results of CCI-779 in breast cancer may question whether mTOR inhibitors work, in part, by downregulating ErbB3. This may be particularly true in those tumors that rely on ErbB3-related pathways for proliferation.

The ErbB receptor family has unique properties. Dimerization between family members recruits various downstream signaling molecules and diversifies the biological response, The type and amplitude of signaling depend upon the co-expression of receptors. Expression of active ErbB2/ErbB3 dimers in breast tumors provide potent mitogenic signals that activate the PI-3K pathway. ErbB3 tyrosine phosphorylation is not merely a consequence of ErbB2 signaling, but rather may be a necessary step to activate the PI-3K pathway. Indeed, aberrant signaling of the PI-3K pathway is associated with breast cancer. Furthermore, tumor cells overexpressing ErbB3 are perhaps dependent on ErbB3 for the activation of PI-3K and mTOR. The mTOR/4EBP1 pathway controls the downstream outcome of PI-3K signaling by supplying critical input regarding protein translation and cell cycle signaling. The inter-dependence of these two pathways is best demonstrated by the ability of rapamycin to display potency against tumors and transformed cell lines with hyperactive PI-3K.

The identification of new components of the regulatory modes in the mTOR signaling circuitry should uncover novel drug targets and help develop new diagnostic and therapeutic strategies. This approach appears promising given an emerging concept that tumors often become dependent upon dysregulated signaling pathways and are therefore hypersensitive to downregulation of these signals (Weinstein, 2002). In this regard, in addition to Herceptin and other ErbB2 targeted therapies, tumors with active ErbB2/ErbB3 dimers may be particularly sensitive to ErbB3-targeted therapeutics, compared to tumors that have upregulated PI-3K/mTOR signaling due to other aberrant signaling molecules. In support of this notion, studies have shown that tumors resulting from overexpression of c-Myc in mice are highly sensitive to even transient inactivation of the oncogene (Jain et al., 2002). Cells that are not dependent on this dysregulated pathway are much less affected by such targeted therapy.

In addition to Akt and PTEN, ErbB3 may serve as a useful prognostic marker. Screening for ErbB3 and components of the PI-3K/mTOR pathway may identify those patients that benefit most from certain treatments. Indeed there is a possibility that cancer cells that overexpress ErbB3 are dependent on an ErbB3-related pathway for proliferation. On that note, ErbB3's essential role in activating the PI-3K/mTOR pathway and driving proliferation of tumor cells provides a model that could explain why

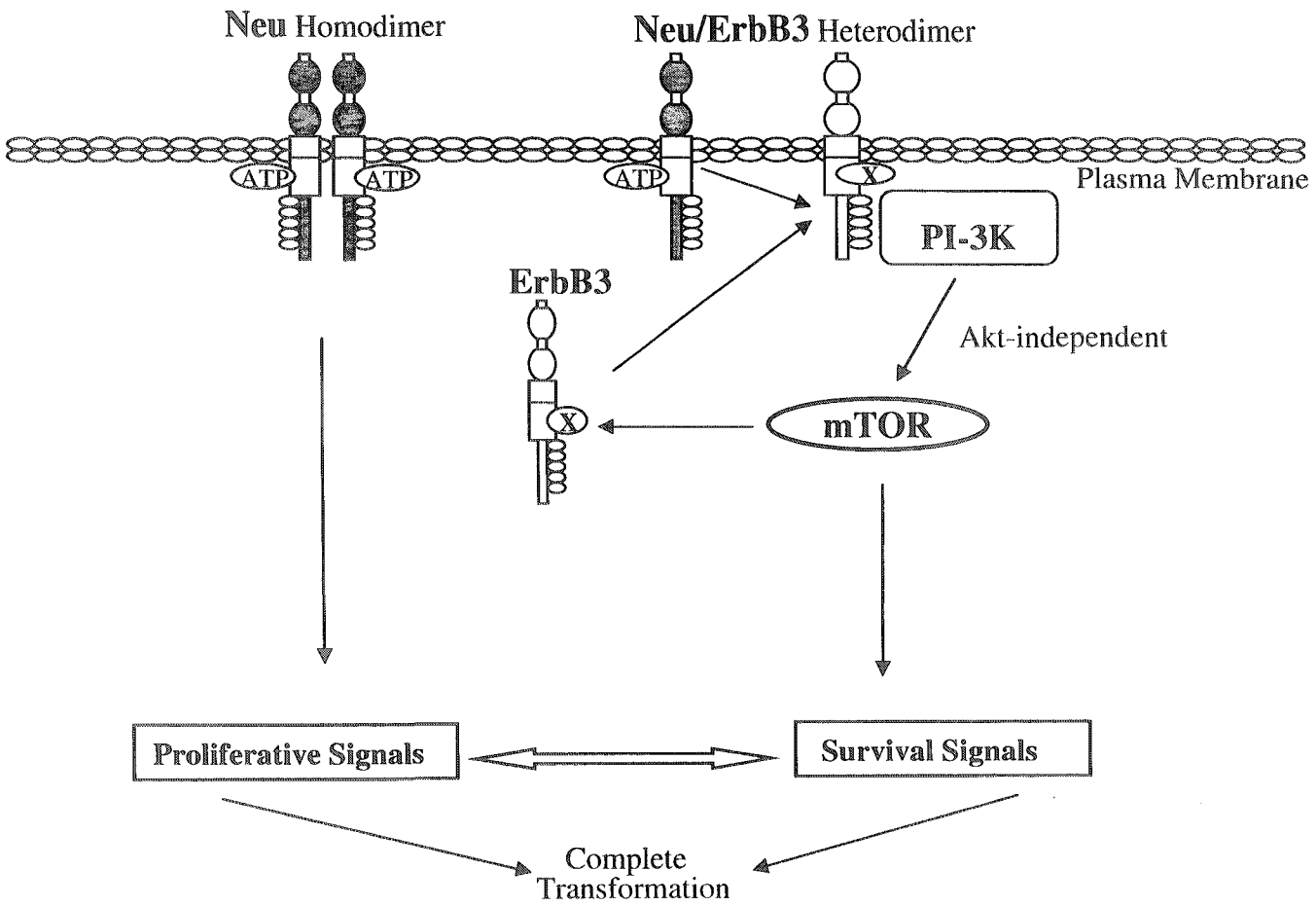
the use of rapamycin in breast tumors overexpressing both ErbB2 and ErbB3 may be beneficial. In addition to clinical trials which establish the effectiveness of CCI-779 in breast cancer, subgroup analyses could determine whether the effectiveness of CCI-779 is most pronounced in those HER2 tumors overexpressing ErbB3.

To maximize response and minimize chemotherapeutic drug resistance, mTOR inhibitors should be used in combination therapy. Clinical trials are often conducted without sufficient molecular insight into the mechanism of response to the intervention. This thesis highlights the potential of mTOR / ErbB3 inhibitor combination therapy, particularly in those breast cancers with activation of PI-3K/mTOR and ErbB3. Although we can only hope for success, this strategy is strikingly similar to recent combination trials of EGFR inhibitors with chemotherapy in advanced stage lung cancer (Dancey and Freidlin, 2003) and the combination trials with CCI-779 and interferon in kidney cancer (Sawyers, 2003a; Sawyers, 2003b). A complete understanding of how mTOR inhibits tumor growth could translate into combination therapy tailored to the biology of the individual tumors. This tailored approach promises to increase the chance of efficacy while minimizing drug toxicity.

Figure 5.1. Model illustrating the conclusions derived from the results obtained in this thesis. This model illustrates the conclusions of the thesis. (1) Both PI-3K and mTOR-dependent phosphorylation of S6K and 4EBP1 are involved in sustaining the high levels of endogenous ErbB3 protein levels by increasing the stability of the protein (2) A major consequence of targeting Neu is dephosphorylation and a decrease in ErbB3 protein as well as members of the PI-3K/mTOR pathway (3) Anti-apoptotic and cell cycle signals provided by PI-3K and mTOR depend, in part, on the ability of phospho-ErbB3 to recruit p85 (4) ErbB3 in conjunction with Neu is involved in the S6K/4EBP1-dependent upregulation of cyclin D1 mRNA and protein in Neu-induced tumors (5) Importantly, inhibition of mTOR in a mouse tumor model delays tumor onset and progression which correlates with a decrease in ErbB3 protein, site-specific dephosphorylation of S6K/4EBP1 and an increase in total hypophosphorylated 4EBP1. (6) Inhibition of ErbB3/p85 interaction decreases transformation and inhibits Neu-mediated tumor growth *in vivo*. (7) Taken together, Neu requires cell cycle and anti-apoptotic signals provided, in part, by ErbB3 for its full oncogenic potential.

Figure 5.1

CONCLUSIONS



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