THE ROLE OF C-SRC IN C-ERBB2/NEU AND ESTROGEN RECEPTOR SIGNALING: IMPLICATIONS IN TRANSFORMATION AND MAMMARY GLAND DEVELOPMENT

By

HAROLD KIM, B.SC. (HONS)

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Doctorate of Philosophy

McMaster University

THE ROLE OF C-SRC IN ERBB2 AND ESTROGEN RECEPTOR SIGNALING

Doctorate of Philosophy (2003) McMaster University
(Medical Sciences) Hamilton, Ontario

TITLE: The Role of c-Src in c-ErbB2/Neu and Estrogen Receptor Signaling: Implications in Transformation and Mammary Gland Development

AUTHOR: Harold Kim, B. Sc Hons. (University of Western Ontario)

SUPERVISOR: Dr. William J. Muller

NUMBER OF PAGES: xvii, 240

Abstract

Breast cancer research has focused on a number of key molecular events suspected to play critical roles in the establishment and progression of the human disease. For example, the c-ErbB2 receptor is amplified or overexpressed in approximately 30% of all human breast cancer cases, its overexpression inversely correlating with a positive patient prognosis. In addition, the estrogen receptor is a key marker in assessing patient prognosis, the progression of the disease state, and is by far the most utilized target to treat breast cancer with approximately 100 million hours of clinical experience with the antiestrogen, Tamoxifen. While these two receptor systems play critical roles in breast cancer progression, the activation of downstream signaling pathways and its consequences on cellular function are unclear. Interestingly, the c-Src tyrosine kinase has been identified to play a role in the modulation of both the c-ErbB2/Neu and the estrogen receptor. Here I will describe a role for c-Src in modulating c-ErbB2/Neu receptor activation and its associated ability to transform at the molecular level, as well as a role for c-Src in estrogen receptor mediated mammary gland development. Significantly, the association of c-Src to the c-ErbB2/Neu receptor plays an important role in the direct and specific activation of the receptor. Furthermore, the loss of c-Src within the mammary gland negatively impacts its development, this delay being mediated via the estrogen receptor. Taken together, these studies demonstrate the importance of c-Src in two receptor systems that have been heavily implicated in development and disease, the estrogen receptor and c-ErbB2/Neu. In both cases, c-Src plays a critical role in receptor activation, coordinating downstream events impinging on reproductive development and in mammary gland transformation. The understanding of the molecular crosstalk between these receptor systems within the mammary gland may provide insight into potential translational therapies in clinical research.

Acknowledgements

"I don't know half of you half as well as I would like, and I like less than half of you half as well as you deserve." J.R.R. Tolkein. The Fellowship of the Ring.

Looking at where I am now, the road that I chose to take ultimately could not have been possible without the support of my supervisor, Bill Muller. In the end, the freedom that Bill provided to carry out ideas and experiments outside of the main goals of the Muller Lab, have taught me independence, insight and scientific rigor. I am a product of his mentorship and I am thankful for the experience. I would also like to thank the members of my committee throughout the years, Drs. Michael Rudnicki, Jim Smiley, Jonathan Lee and Juliet Daniel.

I have always believed that the people surrounding you can positively influence the work that you do and the good friends you forge along the way make the experience all the more enjoyable. I am fortunate to have worked with many of the Muller Lab people even from the early days at McMaster. David Dankort especially is one who has been a great comrade through the years and I am thankful for all his advice and humor. Thanks to Rod Hardy, Bart Maslikowski, Peter Siegel, Marc Webster, Senthil Muthuswamy, Michael Rauh, Valerie Blackmore, and Rob Perry, friends along the way who made the experience all the more enjoyable during those years. As the years grew longer, I am so very thankful to have met Niki Sharan, Michael Laing and John Hutchinson, three people who I truly believe made my stay at McMaster worth every minute. To the three of you, thanks for all the great times, the laughs and endless insight into some of the most bizarre and humorous topics imaginable. I look forward to many more outtakes in the future.

Many thanks to my parents who always believed in me as I walked down the path. Thanks to the faith given to me by the Ashby's and extended families. Most importantly, I owe my total success to my wife Alysa who has been a steady source of support and love from the very beginning. Your help has made me a better and stronger person. Your love has kept me focused and I could not have done it without you. With you I have

realized that this long road traveled in the end is only the beginning, I look forward to spending every minute living life with my family, you and Evelyn. Thank you!

Contribution By Others

I am indebted to a number of talented people who provided me some experimental and technical help. The NeuNT constructs in which the point-mutants, add-backs and the experiment as shown in Figure 3.2 was performed by Dr. David L. Dankort. The quantitative PCR/Lightcycler™ data was performed by Dr. Michael A. Laing. The derivation of the EpH4 cell lines and matrigel assays was performed by Adele Girgis-Gabardo. The mammary gland transplant technique was demonstrated to me by Natasza Kuprios.

Table of contents

List of Figures

List of Tables

List of Used Abbreviations

BSA	bovine serum albumin
CDK	cyclin dependent protein kinases
CHAPS	3-[(3cholamidopropyl)-dimethyl-ammnio]-1-propanesulfonate
CSF-1	colony stimulating factor-1
DCIS	ductal carcinoma in situ
DTT	dithiothreitol
ECL	encanced chemiluminescence
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERα	estrogen receptor alpha
Erk	extracellular regulated kinase
Grb	growth factor receptor bound protein
GST	glutathione S-transferase
HRP	horseradish peroxidase
IB	immunoblot
IP	immunoprecipitation
IPTG	isopropyl-b-D-thioglactopyranoside
LTR	long terminal repeat
mAb	monoclonal antibodies
MAPK	mitogen activated protein kinase
MEK	mitogen activated kinase kinases
MMTV	murine moloney tumor virus
mT	middle T
NDF	neu differential factor (neuregulin)
NRG	neuregulin
nt	nucleotide
PBS	phosphate buffered saline
PDGFR	platelet derived growth factor receptor
PGK	phopshoglycerate kinase
PI	phosphatidylinositol
PI(3,4,5)P3	phosphatidylinositol (3,4,5) trisphosphate
PI(4,5)P2	phosphatidylinositol (4,5) bisphosphate
PI3'K	phosphatidylinositol 3' kinase
PTB	protein tyrosine binding
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PVDF	polyvinylidene diflouride
PyV	polyoma virus

Rb	Retinoblastoma
RTK	receptor tyrosine kinase
SH2	src homology 2
Shc	SH2 and collagen homology proteins
SOS	son of sevenless
STAT	signal transducers and activators of transcription
TBS	tris-buffered saline

CHAPTER 1
Introduction

The epidermal growth factor (EGF) receptor tyrosine kinase (RTK) family has been found to play consistently a leading role in tumor progression. Indeed, a positive prognosis for a patient with ovarian, prostate or breast cancer has been discovered to correlate inversely with the overexpression and/or amplification of this RTK family. Furthermore, a number of downstream signaling pathways that specifically couples to this receptor family appear also to display evidence of deregulation. A situation then exists where the additive and synergistic accumulation of activating or loss-of-function events leads to the development of a pathological state. While many molecular steps appear to drive each stage of tumor development, one of the leading mutations that are found consistently in tumorigenesis involves the EGF receptor family and their downstream targets. This thesis will discuss three molecules that have been heavily implicated in the establishment and progression of mammary gland transformation, the c-ErbB2 receptor tyrosine kinase, the c-Src protein tyrosine kinase and the steroid receptor family member, the estrogen receptor. Interestingly, all three molecules have been suggested to cross talk amongst each other, the significance of these interactions is unclear with regards to mammary gland development and transformation. Here I will present evidence suggesting that c-Src can regulate two receptor systems that have been both implicated in mammary gland development and transformation, the c-ErbB2/Neu receptor and the estrogen receptor.

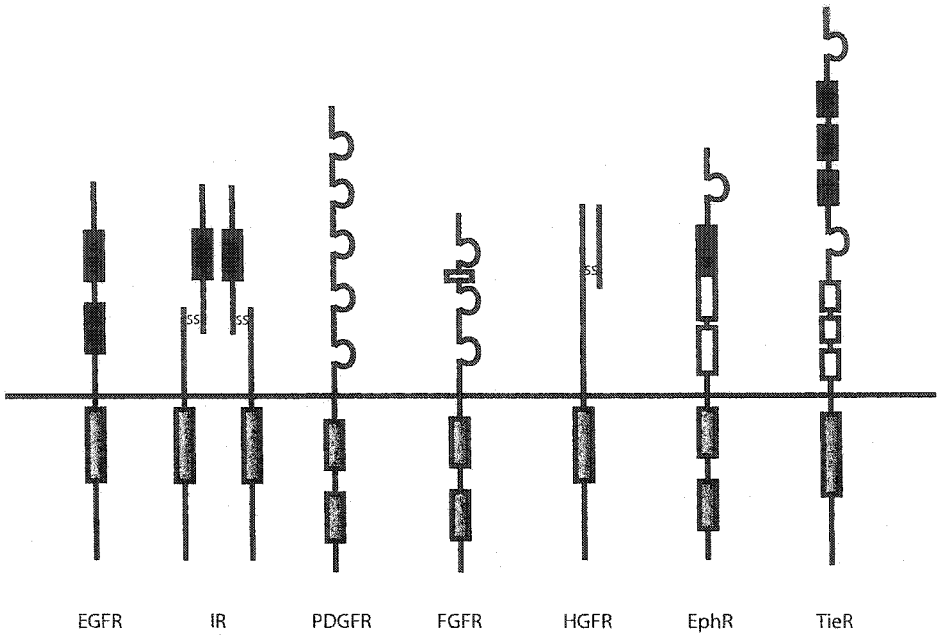
1.1 The epidermal growth factor receptor family of receptor tyrosine kinases

The epidermal growth factor receptor (EGFR) family belongs to the class I receptor group that shares a number of structural characteristics (Figure 1.1). All receptors possess an extracellular region consisting of a ligand-binding domain, two

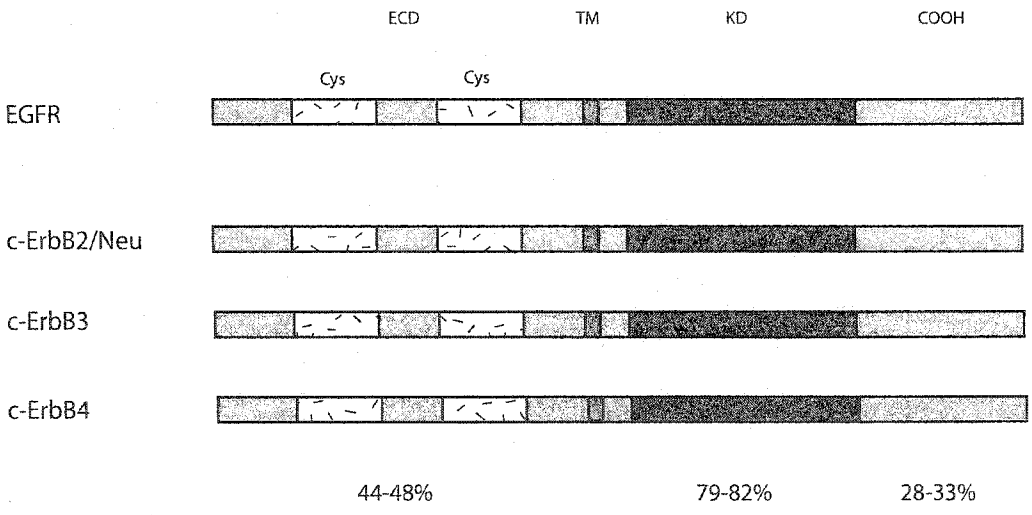
Figure 1.1 Structural landmarks of receptor tyrosine kinases.

(A) Represented are some of the major single-pass transmembrane receptor subgroups (adapted from van der Geer, Hunter and Lindberg, 1994). Each receptor group contains an extracellular ligand-binding domain, a transmembrane region, and a cytoplasmic domain. Some of the features found on these receptor families include cysteine rich regions (black solid boxes), kinase domains (grey boxes), immunoglobulin-like domains (semi-circles), acid rich regions (white horizontal box) and EGF-like domains (white open boxes), cystene bonds (SS). EGFR (Epidermal growth factor receptor), IR (Insulin receptor), PDGFR (Platelet derived growth factor), FGFR (Fibroblast growth factor receptor), HGFR (Hepatocyte growth factor receptor), EphR (Ephrin receptor), TieR (Tyrosine kinase, immunoglobulin and EGF-like repeat receptor). (B) The EGFR family is represented by four members, all of which contain two cystiene rich domains within the extracellular domain (ECD), a single-pass transmembrane (TM), a highly homologous kinase domain (KD) and a divergent carboxyl terminal tail (COOH). Percentage homology relative to the EGFR for each major domain is shown.

A.


EGFR
IR
PDGFR
FGFR
HGFR
EphR
TieR

B.


ECD
TM
KD
COOH
Cys
Cys
EGFR
c-ErbB2/Neu
c-ErbB3
c-ErbB4
44-48%
79-82%
28-33%

cystine rich regions within the extracellular domain, a single pass transmembrane region, a highly conserved catalytic region and a divergent carboxyl terminal tail. The conserved identity of each region varies between family members with the carboxyl terminal region showing the lowest degree of homology, the extracellular domain being intermediate and the kinase domain displaying the highest degree of homology. While the extracellular and carboxyl terminal tail display homology that do not exceed more than 50% between all members, the kinase domain displays the highest homology with the ErbB2/HER2 RTK at 82% relative to the EGFR (Earp et al., 1995).

Two interesting features are worthy to note in this family. Firstly, while three receptors within this family associate with ligands that show some degree of specificity, the ErbB2/Neu receptor has as of yet no identified ligand. Second, while three receptors display catalytic activity, the ErbB3 receptor is catalytically inert (Guy et al., 1994b).

1.1.1 Epidermal growth factor receptor

The EGFR was the first receptor to be identified to possess kinase activity upon ligand binding (Basu et al., 1984). Its initial discovery as a viral oncogene, an avian erythroblastosis virus isolated from erythroleukemias in chickens, led to the identification of its proto-oncogenic counterpart (Downward et al., 1984). Since its identification the v-*erbB* oncogene with its cellular homolog, the 170kDa epidermal growth factor receptor tyrosine kinase has been heavily implicated to play a major role in cellular transformation. Indeed, the overexpression of the EGFR in NIH 3T3 cells confers an EGF-dependent transformation phenotype (Di Fiore et al., 1987a). Moreover, single chain antibodies that specifically associate with and sequester the EGF receptor can inhibit the tumorigenicity of established human cell lines (Beerli et al., 1994; Wels et al., 1995). Many human cancers have been found to overexpress the EGFR, including glioblastomas (Prigent et al., 1996), squamous cell carcinomas of the lung (Garcia de Palazzo et al., 1993), cervix, ovaries and breast (Bauknecht et al., 1989), esophageal (Itakura et al., 1994) and bladder tumors (Lipponen and Eskelinen, 1994). Furthermore,

the overexpression of the EGFR strongly correlates with a poor prognosis of the patient (Harris et al., 1992).

Six specific ligands have been identified to mediate the activation of the EGF receptor. While three shows specificity to the EGFR; EGF, TGFα and amphiregulin, the rest display specificity to both EGFR and c-ErbB4; heparin-binding EGF-like growth factor, betacellulin and epiregulin (Riese and Stern, 1998). In many tumors derived from EGFR overexpression, a concomitant increase in one or more of the ligands has also been observed.

1.1.1a *EGFR and its ligands: Mouse models of tumorigenesis*

While accumulating evidence suggests strongly that the EGFR can play a role in mammary gland tumorigenesis, only recently has its biological significance been addressed. With the generation of a mammary gland specific transgenic mouse for the EGFR, it was observed that these mice develop mammary gland hyperplasias that develop into overt adenocarcinomas in multiparious females (Brandt et al., 2000). Conversely, the overexpression of a dominant negative version of the EGFR results in the inhibition of pubertal mammary gland development (Xie et al., 1997). Consistent with the above observations, transgenic mice that express TGFα driven by the mouse mammary tumor virus promoter (MMTV) develop a range of morphological abnormalities that include lobular and cystic mammary gland hyperplasias, adenomas, and adenocarcinomas that develop with long latency (Matsui et al., 1990). The timeframe in which the tumors arise suggest that TGFα alone may not be sufficient in mediating mammary gland transformation.

One likely partner in potentiating EGFR mediated transformation is the ErbB2/Neu RTK. Indeed, many human cancers express high levels of ErbB2/Neu in conjunction with the EGF receptor. Furthermore, efficient transformation of cells will occur following the overexpression of both ErbB2/Neu and the EGF receptor (Kokai et al., 1989). Recent *in vivo* data suggests that tumor onset mediated by an oncogenic version of Neu can be delayed when in the Waved-2 genetic background (Gillgrass and

Muller, unpublished results). The Waved-2 mouse harbors a naturally occurring EGFR mutation that debilitates its catalytic activity (Luetteke et al., 1994), suggesting that the EGFR plays an important role in Neu mediated tumorigenesis. To further address the possibility of synergy, MMTV TGFα:Neu bitransgenic mice were generated in order to observe the effects of TGFα and its binding partner, the EGF receptor, in ErbB2/Neu mediated tumorigenesis. To this end, tumor formation was found to be accelerated greatly in the bigenics compared to the TGFα or ErbB2/Neu transgenics alone. Furthermore, unlike the activating deletion mutations found prevalent in the ErbB2/Neu transgene (Siegel et al., 1994; Siegel and Muller, 1996) no mutations were found in the transgene of ErbB2/Neu in tumors derived from the bigenic animals (Muller et al., 1996). The acceleration in tumor formation can be reversed with the inhibition of the EGFR in these bitransgenics, again suggesting functional synergy between these receptors in mediating mammary gland tumorigenesis (Lenferink et al., 2000).

In addition to TGFα, the EGF-like ligand amphiregulin has also been found to play a potential role in ErbB2/Neu induced tumors. For example, observations show that amphiregulin is found to be overexpressed 10-fold in a subset of ErbB2/Neu induced tumors (Niemeyer et al., 1999). However, the direct consequence of tumor formation in the presence of amphiregulin remains to be seen. This can potentially be addressed by inhibiting amphiregulin and again monitoring tumor development in ErbB2/Neu induced tumors. Interestingly, TGFα transgenic mice also display an increase in amphiregulin levels (Niemeyer et al., 1999), this increase potentially participating in the acceleration of the tumor kinetics observed in MMTV TGFα:Neu bigenics. These observations underscore the complexity of transgenic systems in that single gene overexpression is more than likely accompanied with multiple molecular consequences, these cumulative effects contributing to the tumor phenotype.

1.1.2 ErbB2/Neu receptor

Historically, the ErbB2/Neu receptor tyrosine kinase was first identified using the carcinogen ethylnitrosourea (ENU) on rats in a model for carcinogenesis (Ohgaki et al.,

1993). To this end a 185kDa phosphoprotein was identified that displayed a high degree of homology to the v-*erb*B/EGF receptor (Downward et al., 1984), suggesting strongly that p185 represented a single-pass cell surface receptor. The presence of p185 directly correlated with the incidence of neuro/glioblastomas in these rats which was confirmed with transfection studies and tumor transplants into syngenic mice (Ohgaki et al., 1993; Perantoni et al., 1987). The generation of antibodies directed to the p185 protein led to the identification of what is now known as the gene *neu* (Drebin et al., 1984; Schechter et al., 1984). Significantly, the catalytic activity of Neu derived from the neuro/glioblastomas appeared to function independent of ligand. Analysis found a single point mutation within the transmembrane region of the receptor. This mutation was specific in that a substitution of a V to E (Valine to Glutamic acid) was sufficient to induce transformation. No other mutations were identified within the cDNA. The significance of the point mutation was later elucidated to confer stability to the receptor via hydrogen bond formation between the receptor dimer (Sternberg and Gullick, 1989; Sternberg and Gullick, 1990; Weiner et al., 1989).

The ErbB2/Neu RTK has been identified in a number of human cancers, the tissues predominantly being epithelial in origin such as gastric, esophageal, salivary, colon, bladder and lung. The focus of research has been on breast and ovarian cancers. Indeed, studies have identified that approximately 30% of all breast cancer cases display an overexpression of this receptor correlating with the poor prognosis of node negative women. Moreover, between 40-60% of Ductal Carcinomas In Situ (DCIS) overexpress ErbB2 (Liu et al., 1992). While the correlation between DCIS and ErbB2 overexpression is significant, the correlation of DCIS developing into overt invasive tumors and the presence of ErbB2 is unclear (Earp et al., 1995).

1.1.2a *ErbB2/Neu: Models of transformation and tumorigenesis*

Although the data suggests strongly that the ErbB2/Neu RTK can play a significant role in the development of mammary tumors, this does not imply a direct or causal role for this receptor in breast cancer. With the use of transgenic mice that

overexpress the point-activated version of Neu (NeuNT) specifically in the mammary epithelium, the question of whether ErbB2/Neu is sufficient to induce transformation in the mammary epithelium can now be addressed. Indeed, the NeuNT RTK driven by the MMTV promoter induces multifocal adenocarcinomas with rapid onset suggesting strongly that the ErbB2/Neu RTK can play a direct role in the formation of mammary tumors (Muller et al., 1988). However, in human breast cancer samples, no evidence of an equivalent point mutation in the ErbB2 transmembrane region was found. The absence of a similar point mutation in ErbB2 may be due to a requirement to convert two base pairs in order to generate the Val to Glu mutation in c-ErbB2 versus one mutation in Neu. Nevertheless, the lack of the transmembrane mutation suggests that other molecular mechanisms provide the activating event.

Given the fact that an equivalent transmembrane mutation is not observed in human samples, one possibility may be that overexpression is sufficient to induce transformation, independent of a secondary activation event. To address the physiological importance of wild-type Neu/ErbB2 overexpression, transgenic mice driven by the MMTV promoter were generated. In contrast to the NeuNT transgenic mouse, focal tumors arose with long latency. Histological evaluation of the wild-type Neu/ErbB2 transgenic mammary gland revealed normal mammary epithelium juxtaposed to transformed epithelium, in spite of detectable transgene levels in both tumor and normal adjacent mammary glands. This observation suggests that overexpresion was not sufficient for transformation and that other activating events were required to confer the full transforming potential of the Neu RTK (Guy et al., 1992).

The increase in tumor latency observed in the wild-type Neu/ErbB2 transgenic mouse might be due to a requirement for secondary mutations in addition to overexpression. Upon further analysis of the MMTV Neu/ErbB2 RNA transcripts derived from the tumors of the transgenic mice, no mutations were discovered within the transmembrane region. However, many of the RNA transcripts did suffer from in-frame deletions that localized themselves to the juxtamembrane portion of the extracellular domain (ECD) of the receptor (Siegel et al., 1994). These Neu deletions (NDL), when

assessed for transformation potential displayed various degrees of phenotypic severity. Importantly, all of the deletions altered the cysteine rich region found in the juxtamembrane region of the ECD, this region having been implicated to play a role in receptor dimerization upon ligand binding. Furthermore, these deletions were identified to be sufficient in mediating transformation both *in vitro* and *in vivo* (Siegel and Muller, 1996). Normally, in the absence of ligand, collisions between receptor monomers occur frequently that do not result in the activation of intracellular signaling pathways (Jiang and Hunter, 1999). Presumably, in the presence of ligand the collisions observed between receptor monomers become stable by ligand binding. While c-ErbB2/Neu does not have a ligand, the presence of the altered cysteine residues does form intermolecular disulfide bonds generating a constitutively active receptor complex independent of ligand. Indeed, the disruption of the cystine bonds with β-mercaptoethanol prevents transformation as assessed by focus formation (Siegel and Muller, 1996). With the generation of transgenic mice that harbor the most potent NDL variant as assessed in transformation assays, it was found that tumors arose focally with a T_{50} of 143 days, suggesting that the role of cysteine residues in the ECD play an important role in receptor dimerization. Therefore, the loss of cysteine residues as a result of somatic mutations concomitant to receptor overexpression appears to lead to focal mammary epithelial cell transformation (Siegel et al., 1994).

Although the above evidence suggests strongly that extracellular mutations involving cysteine residues can result in receptor activation via constitutive dimerization, it is unclear whether these deletions are of any relevance to the molecular mechanism involved in the development of the human disease. To this end, a number of primary breast tumor samples were analyzed to identify similar ECD deletions that may possess an activating role. Interestingly, in addition to the high levels of ErbB2 in these human breast tumor samples, a great majority of them harbored deletions in the ECD proximal to the transmembrane region (Siegel et al., 1999). Significantly, this deletion removes a full exon while maintaining the reading frame of the mutant human ErbB2 receptor. The mutant ErbB2 RTK approximately represents 5% of the endogenous ErbB2 levels as

assessed by RNase protection. While the identification of the mutant ErbB2 RTK is intriguing, the role of the truncated RTK is still unknown. Outstanding questions include the functionality of the mutant ErbB2, the role of the mutant ErbB2 in tumor formation and its mechanism of action. Studies to address directly the importance of the exon deletion will greatly aid in the identification of its significance.

1.1.2b *Signal transduction from ErbB2*

The accurate translation of an extracellular signal to elicit an appropriate cellular response is dependent on the precise and timely association of specific substrates to the receptor itself, resulting in the transmission of an intracellular signal with high fidelity. In addition to the EGFR, the c-ErbB2/Neu RTK is believed to propagate its' signal via five autophosphorylation sites that reside within the carboxyl terminal region of the receptor. Upon activation, receptor autophosphorylation presents high affinity binding sites for a number of proteins that can dock directly to the phosphorylated residue, for example the adaptor proteins Shc and Grb2 (Rozakis-Adcock et al., 1993; Rozakis-Adcock et al., 1992). Direct evidence however implicating the importance of similar sites on the c-ErbB2/Neu RTK was lacking. To address the significance of these autophosphorylation sites in transformation, a systematic mutagenesis approach was undertaken whereby the ability of each tyrosine to associate with their substrates upon either their loss or in isolation was assessed. Significantly, it was discovered that the autophosphorylation sites appear to be functionally redundant when assessed by an in vitro transformation assay. Furthermore, these redundant pathways appear to activate the Ras/MAPK pathway via multiple molecular mechanisms. Interestingly, out of the five sites site A (Y1024) appears to play a negative role in transformation (Dankort et al., 1997).

Similar to the EGFR, it was found that the ability to activate the Ras pathway is dependent on the association of specific adapter proteins to distinct autophosphorylation sites found on c-ErbB2/Neu. The Grb2 adapter protein associates directly with YB (Y1144) while the Shc adaptor protein associates with site D (Y1227). DNA synthesis as initiated by the activation of the Ras pathway can be inhibited from sites B, D and E

(Y1248) with the use of antibodies against Ras. Consistent with this observation, transformation mediated by NeuNT can be attenuated with the addition of the protein Rap1A, a downstream competitor for Raf (Dankort et al., 2001; Dankort et al., 1997). Interestingly, while DNA synthesis mediated by YC (Y1201) is refractory to an anti-Ras neutralizing antibody (Y13-259), it is not negatively affected by Rap1A, a Ras antagonist believed to neutralize Ras by sequestering Ras targets. This suggesting that multiple mechanisms are utilized by these autophosphorylation sites to activate the Ras pathway (Dankort et al., 2001).

1.1.3 Receptor activation

The leading hypothesis for type I receptor activation involves the binding of two monomeric ligands to the receptor, which induces the formation of receptor dimers leading to the transphosphorylation of specific residues within the carboxyl terminal region. The phosphorylation of specific residues within the carboxyl terminal tail generates high affinity binding sites for a number of proteins that contain protein-protein interaction domains that mediate receptor-substrate complex formation. Evidence has suggested that these receptors undergo many rapid and transient collisions with each other, however full activation of the receptor is not achieved until the receptor complex is stabilized by a combination of ligand occupancy or from intracellular factors. Previous data has suggested that the receptor complex can be stabilized with the association of one monomeric ligand that can simultaneously bind two receptor molecules (Tzahar et al., 1997). Recently, single molecule tracking has demonstrated that a monomeric EGF molecule associates with a receptor dimer, which then recruits a second monomeric EGF molecule to initiate receptor activation (Sako et al., 2000).

Models for the activation of the EGFR family revolve around the necessity for dimer formation in order to initiate signal transduction. While models have been generated that involve homomeric receptors, the most studied being the EGFR, it is known that within the EGFR family, heterodimerization can occur that is dependent on ligand occupancy within the dimer. This is complicated by the fact that the c-ErbB2/Neu

receptor has no specific ligand. Since these receptors are believed to be dimeric, how do ligand mediated dimers form between the c-ErbB2 receptor and other members of the family? Two models have been investigated to explain this. The first, referred to as the bivalency hypothesis, states that there exists two sites of association for a ligand on each receptor. For example, with a c-ErbB2/c-ErbB3 (B2/B3) heterodimer the high-affinity binding site would be with the B3 receptor for NRG class ligands. The low affinity site would obligatorily be on the B2 receptor for the same ligand (Tzahar et al., 1997).

Another possibility stems from data that suggests that the NRG class of ligands have absolutely no affinity to c-ErbB2 (Ferguson et al., 2000). Given this observation, biophysical analysis on receptors within this family suggests that receptor multimerization, not simple dimerization, occurs upon receptor activation. Furthermore, it has been hypothesized that multimerization upon ligand binding favors homomeric associations with the specific ligand class i.e. EGF promotes EGFR dimers, NRG's promotes c-ErbB3 or c-ErbB4 homodimers. Thus, ligand binding results in multimer formation of at least four monomers consisting of two dimers (Lemmon et al., 1997; Ferguson et al., 2000). This may explain why it is possible for a kinase negative EGFR to be able to activate c-ErbB2 upon EGF binding (Spivak-Kroizman et al., 1992), as well as how any heteromer formed with ErbB3 can become active. While the multimerization hypothesis provides an explanation for the monomeric ligand-induced transphosphorylation event, it does not shed light on the mechanism of c-ErbB2 dimer formation within the multimer upon EGF mediated activation of a receptor complex. It may be possible that c-ErbB2 may not need a ligand to achieve receptor complex activation. This also implies that the extracellular conformation of ErbB2 may obviate the requirement of a ligand and that oligomerization is the only necessity for complex activation. Recently the crystal structure of the ligand-binding domain of the EGFR has revealed some intriguing findings. It appears that when EGFR homodimers form, the ligand binding regions face 180 degrees away from each other. Therefore, this structure does not appear to be conducive to a bivalent ligand-binding hypothesis where one ligand monomer forms a bridge between two receptor dimers. The crystal structure of the

ligand-binding region lends itself more to the multimerization hypothesis, yet this evidence does not advance the multimerization hypothesis either. Given this observation, it will be interesting to compare the structural features of this receptor family when the solution structures of the ErbB2 receptor as well as the remaining family members are generated.

The alignment of the alpha helical extracellular region has been implicated to play a role in establishing a conformation optimal for receptor signaling since the substitution of cysteine residues along the receptor-receptor interface leads to differences in dimerization and transformation efficiency (Burke and Stern, 1998). With regards to tumorigenesis, deletions in the extracellular region that can disrupt these cystine residues have been shown to activate the receptor and induce constitutive dimerization and activation (Muller et al., 1996). Moreover, differences in the position of the deletions can modify the transforming potential of the receptor (Siegel et al., 1994). The transforming ability observed in the Neu RTK upon mutation of a single amino acid from a valine to a glutamic acid residue at position 664 within the transmembrane region further argues the importance of structure in the regulation of receptor activity (Bargmann et al., 1986; Bargmann and Weinberg, 1988a; Cao et al., 1992; Gullick et al., 1992).

Receptor proximity appears to be sufficient in the initiation of receptor activation. Given this, how do these collisions activate the kinase regions of the receptors? It is possible that conformational changes that affect the kinase domain occur upon close proximity between receptors, exposing critical sites within the domain and initiating kinase activity (Jiang and Hunter, 1999). Indeed, it has been suggested that the activation loop is quite flexible, alternating in its conformation from "closed" to "open". It is possible that oligomerization of receptors in the presence of a ligand can stabilize the activation loop in an "open" conformation long enough for ATP to insert itself into the binding pocket and initiate transactivation (Hubbard et al., 1998). Receptors such as the insulin or FGF receptor harbor specific tyrosines within the activation loop of the kinase domain that are required to be phosphorylated in order to achieve full receptor activity (Ellis et al., 1986; Mohammadi et al., 1996). Alternatively, other kinases within the

receptor complexes may use the receptors themselves as substrates, the association of the receptor with the ligand may act more as a stabilizer to provide enough time for cytoplasmic proteins to initiate signal transduction. Indeed, the inhibition of protein phosphatases induces receptor activation independent of ligand binding (Posner et al., 1994) suggesting that proteins do use receptors as a substrate and are negatively regulated by phosphatases. It is clear that ligand occupancy plays a role in the stabilization of receptor oligomers, however the stoichiometry of the receptors within the complex appears to be in question (Lemmon et al., 1997; Tzahar et al., 1997). Furthermore, receptor activation appears to generate changes in receptor conformation however the final conformation of the activated complex is unclear (Jiang and Hunter, 1999). Is there a requirement for specific residues to be phosphorylated for optimal activation? Is the conformational change generated by the formation of the receptor dimer/oligomer sufficient in mediating ATP loading and subsequent catalytic activation of the receptor? Analysis of the IR and the FGF receptors do suggest that both the phosphorylation of specific residues and the resulting conformation upon activation is important. Moreover, it is unclear whether specific cytoplasmic proteins play a role in potentiating the activation of the receptor.

1.2 Molecular specificity displayed by the EGFR family

The ability of this receptor family to generate such specificity and diversity in signaling has been attributed to its ability to operate at various levels of interaction. Thus, at the molecular level a number of options exist that increases the signaling capacity of the EGFR family. These include (1) ligand specificity, (2) combinatorial dimerization, (3) catalytic specificity and (4) substrate specificity.

1.2.1 Ligand specificity

The ligands for the EGF receptor family are classified into groups based on their association to specific receptor family members. The EGFR has six specific ligands, EGF, TGFα, amphiregulin, heparin-binding EGF-like growth factor, betacellulin and

epiregulin. Three ligands that associate specifically with ErbB3, Neu differentiation factor (NDF)/heregulin, glial growth factor (GGF) and acetylcholine receptor inducing activity (ARIA) are products of one gene which is referred to as the neuregulins (NRG-1) (Normanno et al., 1994; Riese et al., 1996).

It is clear that receptors upon activation by various EGF-related peptides undergo homodimerization as well as heterodimerization. Using various combinations of the EGF receptor family members' transfected into NIH 3T3 cells a number if interesting observations were noted. Specifically, cytoplasmic substrates such as Shc and Grb2 associate to specific receptor family members depending on the EGF-related peptide. For example, Shc and Grb2 only appear to associate upon EGF stimulation but not in the presence of Neu differentiation factor (NDF) ligand (Olayioye et al., 1998). Furthermore, the rate of internalization varied depending on the ligand used; in this case, EGF results in the rapid internalization of the EGFR, in contrast to the delayed internalization observed in NDF stimulated EGFR. In addition, depending on the receptor partners within the heterodimer, specific combinations of associated cytoplasmic proteins are observed, such as Shc, Grb2 and PI3'K (Olayioye et al., 1998). Consistent with previous observations where a specific kinase domain phosphorylates specific autophosphorylation sites, it is now apparent that the association of specific dimerization partners will influence the pattern of autophosphorylation on each receptor translating into different signal output. It is still unclear how the receptors modify the association of the substrates to the receptor, does the kinase domain of each receptor modify the others activity and specificity? If so, does this translate to differences in the direct phosphorylation and possible activation of their cytoplasmic targets?

1.2.2 Catalytic specificity

The kinase regions of the EGF receptor family can be divided into two distinct domains, referred to as TK1 and TK2. The former domain from amino acid 657 to 808 within the kinase region shares a high degree of homology between all of the EGF receptor family members as well as housing the ATP binding site. The TK2 region from

amino acid 809 to 927, which houses the catalytic activation loop, is less conserved than the TK1 region (Di Fiore et al., 1992; Di Fiore et al., 1990). The molecular model for full catalytic activation consists of an activation loop modulating the accessibility of ATP into its binding pocket, much like a molecular gate. A phosphotyrosine residue that is conserved within the activation loop of the EGFR and c-ErbB2 appears to play a role in modulating the catalytic activity of this receptor family. However, the importance of this tyrosine in modulating receptor activity appears to differ. For example, in the in the c-ErbB2/Neu receptor a mutation of this tyrosine to a phenylalanine drastically reduces its catalytic activity as measured by substrate phosphorylation (Zhang et al., 1998). In contrast, a similar mutation on the EGFR does not alter its kinase activity (Gotoh et al., 1992; Hubbard et al., 1998).

Differences in the catalytic specificity of each kinase region have been suggested from the observation that a specific pathology correlates with the specific overexpression of an EGF receptor family member. For example, the overexpression of the EGF receptor correlates with the presence of squamous cell carcinomas while the overexpression of the ErbB2/Neu receptor correlates with the formation of adenocarcinomas and neuro/glioblastomas (Lovekin et al., 1991; Merlino et al., 1984; Paterson et al., 1991; Slamon et al., 1989; Xu et al., 1984). Furthermore, in transformation assays ErbB2/Neu is 100-fold more potent in its transforming ability when compared to the EGF receptor (Di Fiore et al., 1987a; Di Fiore et al., 1987b). These biological differences resulting from the overexpression of a specific receptor may be due to subtle differences in the specificity of the kinase region. To this end, it was discovered via chimeric receptor studies between ErbB2 and the EGFR that catalytic specificity resides in the TK1 domain of the kinase region. Indeed, the constellation of associated proteins differed between each chimeric receptor (Di Fiore et al., 1992; Di Fiore et al., 1990), underlying the importance of substrate specificity mediated by each kinase region.

Although specificity appears to contribute to differences in signaling, what is its biological significance? The ability of various kinase regions to produce specific biological/pathological readouts was tested using chimeric molecules of the EGFR and

the c-Src protein tyrosine kinase. While the deregulation of the *erbB* gene has been associated with leukemia's, oncogenic *src* has been associated with sarcomas. With the generation of an EGFR that harbors the c-Src kinase region, this chimeric now converts the leukemogenic *erbB* gene into a sarcomagenic *src* gene (Chang et al., 1995). Interestingly, autophosphorylation and substrate phosphorylation patterns of the ErbB/Src chimera differed from the parental protein tyrosine kinases suggesting that other regions of the ErbB and Src molecules influence the phenotypic/pathological output of these genes. The Ret receptor tyrosine kinase has been implicated in the causal development of multiple endocrine neoplasia type 2B, a result of a single point mutation in the Ret receptors kinase region. In order to address the role of kinase specificity in the development of specific human diseases, a homologous point mutation was introduced in the EGF receptor (Pandit et al., 1996). Interestingly, the ability of the mutated EGF receptor to transform was enhanced. Furthermore, using an oriented library approach it was discovered that the substrate specificity of the mutated EGF receptor was altered from the wild-type EGF receptor (Pandit et al., 1996). Similar observations were reported in an EGF receptor/ErbB2 chimera in that the transformation ability and the profile of phosphotyrosine proteins associated with the EGFR/ErbB2 chimera differed from their parental receptors (Di Fiore et al., 1992). These lines of evidence suggest strongly the importance of the kinase region in mediating specificity and general receptor signaling.

1.2.3 Substrate specificity

The association of various cytoplasmic signaling molecules to the EGF receptor family is dependent on the phosphorylation of specific amino acids found within the carboxyl terminal region of the receptor. These phosphorylated amino acid residues, most relevant to this discussion being tyrosine, can create high affinity binding sites for a number of protein domains that directly interact with the phosphotyrosine, in turn initiating a signaling cascade. Many of these domain structures have been found to act autonomously, the same protein interaction domain being found in a number of proteins with vastly different functions. Evolutionarily speaking, it has been speculated that

protein function can in this way adapt to various signaling requirements with either the addition or loss of domain structures, therefore potentially altering the localization and function of the protein to meet the need of the cell.

Domain structures that mediate the association of proteins to their respective targets can be classified by their recognition motifs. The current list of motifs include SH2, PTB, SH3, WW, EVH1, PH, FYVE, PDZ, and EH domains (Sudol, 1998). SH2 or PTB containing proteins recognize a core tyrosine that is flanked by amino acid sequences directing the specificity of that residue to a specific domain. For example, the SH2 domain from c-Src optimally recognizes the motif (p)YEEI, the SH2 domain from the p85 subunit of PI3'K recognizes the motif (p)YxxM. The PTB module recognizes the motif NPxY, which interestingly has the ability to recognize both a phosphorylated and a non-phosphorylated tyrosine, the latter being thought to have evolved prior to the former PTB domain, possibly to increase the repertoire of this signaling module (Pawson and Nash, 2000). For example, the FRS2 PTB domain on one hand recognizes NPx(p)Y on the TrkA receptor, while on the other hand recognizes NPxY on the FGF receptor (Dhalluin et al., 2000). EVH1, WW and SH3 domains have been found to recognize proline rich motifs (Fedorov et al., 1999; Macias et al., 1996; Yu et al., 1994), SH3 domains in particular recognizing a core consensus sequence of PxxP with flanking sequences providing additional specificity to the module. SH3 domains contain two hydrophobic pockets that recognize the core sequence and associates to this motif in either an amino-to-carboxyl terminal orientation (class I), or in the opposite orientation (class II) (Feng et al., 1994). FYVE and PH domains recognize modified lipid moieties derived from phosphoinositides (Hurley and Meyer, 2001), thereby generating a mechanism of localization for PI kinases to cellular membranes that result in changes in cell behavior. PDZ domains recognize short amino acid sequences at the extreme carboxyl end of a protein (Morais Cabral et al., 1996) while EH domains (de Beer et al., 1998) recognize Asn-Pro-Phe sequences that are typically associated with proteins found in protein trafficking.

1.3 The c-Src protein tyrosine kinase

The c-Src protein tyrosine kinase was initially described as a transforming oncogene derived from avian sarcomas (Stehelin et al., 1976a; Stehelin et al., 1975; Stehelin et al., 1976b; Varmus et al., 1973). This transforming retrovirus, the Rous sarcoma virus (RSV) has been the most heavily studied protein tyrosine kinase to date and has been the prototypical tyrosine kinase in signaling. A major advance early in this field was the discovery that v-Src displayed kinase activity suggesting that this molecule can play an active role in signal transduction (Hunter and Sefton, 1980; Sefton et al., 1980). The Src protein tyrosine kinase family falls within 52-62 kDa in size and contain contains 10 members identified by similar structural features. Expression patterns of these members display both a ubiquitous and tissue specific expression pattern. For example, Src, Yes and Fyn all appear to be expressed in the majority of tissues while Blk, Fgr, Hck, Lck and Lyn appear to be predominantly found in hematopoietic cells.

1.3.1 Domains and structure of c-Src

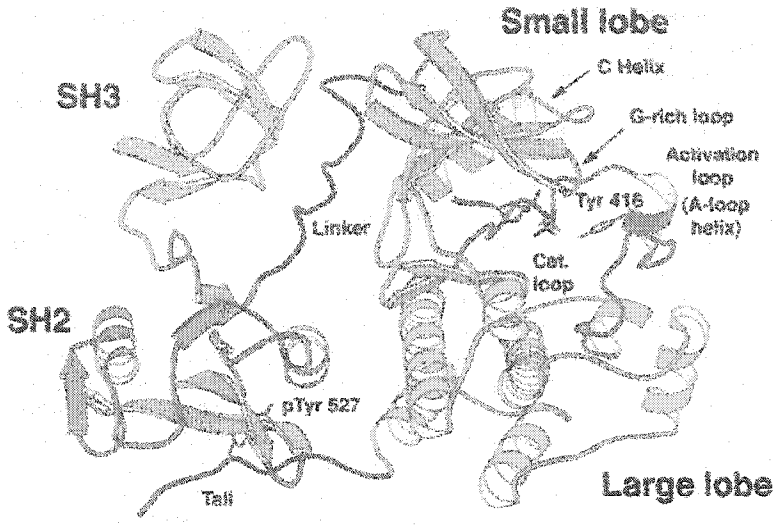
A significant discovery regarding c-Src was its modular structure. These domains are highly conserved throughout evolution, appear to be functionally autonomous and have evolved to work together to regulate both intra- and intermolecular protein associations as well as its own catalytic activity (Figure 1.2). Six regions defined by sequence and function exist within the Src PTK family: (a) the SH4 domain (b) a unique region, (c) a SH3 domain, (d) a SH2 domain, (e) a kinase domain and, (f) the negative regulatory region.

SH4 domain: Experiments have shown that the transforming ability of v-Src is dependent on its association to the cytoplasmic lipid face of the cell. Within the first 14 amino acids of all Src family members, the most notable amino acid is at position 2. Here, myristic acid is covalently bound to Gly-2 and mediates the association of all Src members to the inner plasma membrane (Cross et al., 1984). The consensus motif for this event is not clear, however it does appear to at least encompass amino acids 1-7. Indeed, the efficient myristylation of a fusion protein is achieved when amino acids 1-7 of c-Src

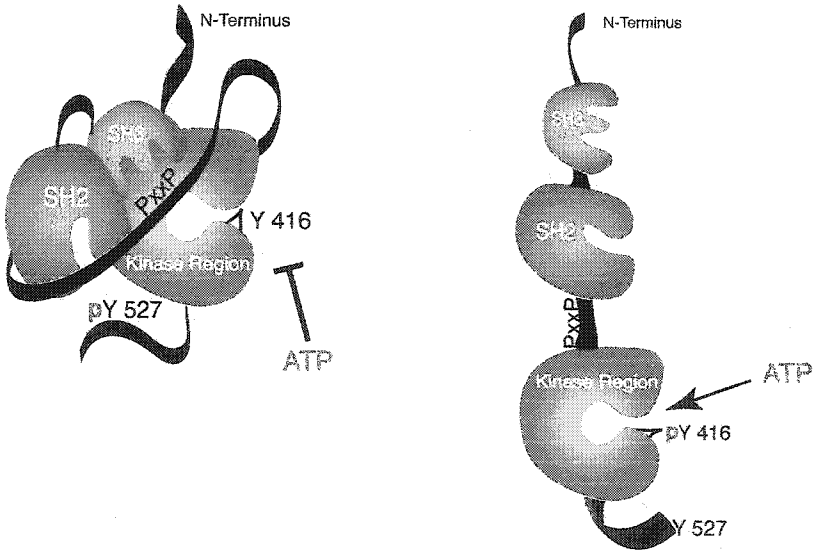
Figure 1.2 Structural representation of c-Src.

(A) Crystal structure of the c-Src protein tyrosine kinase in the closed inactive conformation. Shown in yellow, green, dark blue and light blue are the SH3, SH2, small lobe and large lobe of the kinase domain respectively. The proline linker region is colored red, the activation loop region purple and the catalytic loop gold. Figure adapted from (Sicheri and Kuriyan, 1997). (B) Graphic illustration of the mechanism of c-Src regulation. In the fully closed and inactive conformation, both the SH2 and SH3 domains associate with the regulatory phosphotyrosine 527 and the proline residues found within the linker region of the molecule respectively. The fully open and active conformation is observed when both domains disassociate intramolecularly.

A.


Small lobe
C Helix
SH3
G-rich loop
Activation loop
Tyr 416
(A-loop helix)
Linker
Cat. loop
SH2
pTyr 527
Tail
Large lobe

B.


N-Terminus
SH3
SH2
PxxP
Y 416
Kinase Region
pY 527
ATP
N-Terminus
SH3
SH2
PxxP
Kinase Region
ATP
pY 416
Y 527

are linked to the protein pyruvate kinase (Kaplan et al., 1988). This is also true with fusions incorporating amino acids 2-9 of c-Src. However, the myristylation of Gly-2 within the consensus motif is not sufficient for the membrane targeting of the pyruvate kinase/Src fusion protein suggesting that other regions of c-Src are necessary for proper targeting. To this end, flanking residues that are found following Gly-2 have been suggested to influence myristylation efficiency. For example, the conversion of Lys-7 to asparagine decreases myristylation. However conversion to arginine has no effect, suggesting that an overall positive charge may be necessary for efficient myristylation (Kaplan et al., 1988). In addition to myristylation, some Src family members are also palmitylated on serine, threonine, or cystine residues found within the SH4 region (Saouaf et al., 1997). The consequence of this event is unclear however, it is speculated to possibly work in conjunction with Gly-2 to stabilize Src family members to the membrane.

Unique region: The unique region represents a stretch of amino acids 50 to 80 residues in length starting immediately after the SH4 domain. Like the SH4 domain, serine and threonine residues appear to be phosphorylated by specific cellular proteins, which includes Ser-17 by cyclic AMP dependent protein kinase A (Walker et al., 1993), Ser-12 and Ser-48 by protein kinase C (Gould et al., 1985), and Thr-34, Thr-46, and Ser72 by $p34^{cdc2}$ (Kaech et al., 1993). The significance of the phosphorylation of Ser-17 by cAMP may lie in the resulting change in the hydrophobicity of the amino terminal region, correlating with its translocation to the membrane and an increase in kinase activity (Hirota et al., 1988; Walker et al., 1993). Mutagenesis studies did not shed light on the significance of Ser-12 phosphorylation by Protein kinase C as assessed by kinase activity, transformation ability or cellular morphology (Yaciuk et al., 1989). Modification of Thr-34, Thr-46, and Ser72 by cdc2, first characterized in the *Xenopus* model, greatly accelerated meiotic maturation (Spivack et al., 1984; Spivack and Maller, 1985).

SH3 domain: This domain is a modular and functionally autonomous region that can mediate both intra- and intermolecular protein associations. Structural analysis has identified this 50 amino acid stretch to consist of five to eight β strands that form a β

barrel. Hydrophobic regions generated by a number of aliphatic and aromatic amino acids are surrounded by hydrophilic loops, generating a pocket that preferentially recognizes regions that are proline-rich. Indeed, mutational analysis of these hydrophobic residues ablates the ability of the c-Src SH3 domain to form both inter- and intramolecular associations (Pawson, 1994). Consistent with this observation, the transformation capacity of these c-Src mutants are severely attenuated in NIH-3T3 cells suggesting that this domain can regulate transformation via the SH3 domain (Erpel et al., 1995). Whether the loss of the SH3 domain also results in the inability of c-Src to associate with other molecules important in transformation is unknown. A similar result was obtained upon both PDGF and EGF stimulation via microinjection of c-Src SH3 deletion mutants further suggesting the importance of the SH3 domain in c-Src mediated transformation (Erpel et al., 1996). However, other studies show that deletion (Okada et al., 1993), or displacement of the SH3 domain promotes full activation (Moarefi et al., 1997). It is thus possible that while deletion of the SH3 domain can attenuate transformation, the protein itself may be active. This further suggests that transformation may be dependent on specific proteins that associate with the SH3 domain.

SH2 domains: The solution structure for the c-Src SH2 domain reveals two β-sheets flanked by α-helicies on either side, generating a hydrophobic core that serves as the insertion point for the phosphotyrosine. Here, the arginine that is found within a conserved 'FLVRES' motif, which is diagnostic for SH2 domains, contacts the hydroxyl oxygen residue of tyrosine via hydrogen bonds. An arginine located within the first α-helix (α-A) in addition to a lysine on the hydrophobic face of the β-sheet provides additional bond strength between the SH2 domain and the phosphotyrosine by interacting with the amino-ring (Waksman et al., 1993).

Outside of the binding pocket, residues in the C-terminal direction from the phosphotyrosine determine the specificity of the interaction between individual SH2 domains with their targets. While the amino acids that lie in the +1 and +2 positions lie across the face of the domain, the +3 position sinks into another hydrophobic region. Here within the second pocket, the residues that line and interact with the +3 residue

determine specificity (Eck et al., 1993). An optimal binding sequence of pYEEI was elucidated for the Lck SH2 domain by a phosphopeptide library screen (Songyang et al., 1993), this sequence being present in the hamster polyoma middle-T antigen and presenting an extremely strong affinity at approximately 1nM (Payne et al., 1993). Similar disassociation constants were found with other SH2 domains, like that found in the p85 subunit of PI3'K, in conjunction with a rapid turnover allowing for a high rate of exchange between the substrate and the SH2 containing protein (Felder et al., 1993). However, within the literature there are other reports that suggest a weaker association between the optimal peptide and the c-Src SH2 domain. A reassessment of the affinity of the c-Src SH2 domain to various substrates revealed that indeed the initial report might have been off by 2 orders of magnitude (Ladbury et al., 1995) suggesting that these interactions are in fact weaker than previously suggested. Even the carboxyl terminal phosphotyrosine, which does not neatly fit the predicted consensus, forms an intramolecular association with its own SH2 domain and was found to display a disassociation constant in the μM range (Ladbury et al., 1995) and has been described as 'moderate' (Liu et al., 1993).

The specificity mediated by the +3 residue was addressed with the generation of mutant SH2 domains at the EF1 region in both the c-Src and Grb2 SH2 domains. To this end, Thr 215 found in c-Src was exchanged for a Trp from the homologous site in the adaptor protein Grb2. This change in residue resulted in the ability of the c-Src SH2 domain to substitute for the Grb2 domain, initiating Ras signaling *in vivo* (Marengere et al., 1994). Structural analysis of the differential interaction of the mutant SH2 domains shows that the ThrEF1Trp substitution results in the physical occlusion of the +3 pocket, leading to a larger surface area for the +2 residue (Kimber et al., 2000).

Kinase domain: This region is approximately 300 amino acids and is structurally represented by 2 distinct lobes, an N-terminal and a C-terminal lobe. The latter region appears to provide the bulk of the interactions seen by the kinase domain (Sicheri and Kuriyan, 1997). In addition, a 'catalytic segment' interacts directly with the phosphate groups of ATP, and an 'activation segment' harbors tyrosine 416, a target for

phosphorylation resulting in the full activation of the kinase. Tyrosine 416 is the key residue that when phosphorylated, alters the charge and conformation of the region thus promoting full activity (Kmiecik and Shalloway, 1987). In conjunction with the phosphorylation of Y416, both the SH2 and SH3 domains can regulate the activity of the kinase via steric hindrance and impaired flexibility of the two lobes. It appears that while the SH2 domain is critical for the generation of the 'closed' conformation, it is the SH3 domain that plays the dominant role in c-Src activation (Moarefi et al., 1997). When in a 'closed' conformation, the α helix, αC, is rotated outward, displaced by its own SH3 domain. Activation leads to the displacement of the SH3 domain, and the phosphorylation of Y416, which results in the αC helix swinging back into a conformation that favors kinase activity (Sicheri and Kuriyan, 1997).

By using degenerate peptides, the ability of kinases to selectively phosphorylate specific substrates was assessed. To this end, it was found that indeed, different kinases do display preferences in the substrates that they will phosphorylate. Moreover, differences exist between kinases found on receptors versus cytoplasmic proteins. With regards to the latter, cytoplasmic kinases display a tendency to phosphorylate residues within peptide motifs that their own SH2 domain can recognize, thereby promoting an active signaling role by direct association subsequent to activation (Songyang et al., 1993).

Negative regulatory region: This region is approximately 16 to 19 amino acids in length and contains the regulatory tyrosine (Y527) that intramolecularly associates with the SH2 domain resulting in the regulation of c-Src activity. Previous lines of evidence have suggested that Y527 is important in the regulation of kinase activity (Laudano and Buchanan, 1986), which includes the deletion of Y527 and its flanking amino acids in v-*src*, v-*yes*, and v-*fgr*. Furthermore, the conversion of Y527 to phenylalanine leads to an increase in kinase activity and the ability to transform both in cell culture and *in vivo* (Kmiecik and Shalloway, 1987).

Given that kinase inactive forms of c-Src are still phosphorylated at Y527, it was hypothesized that another kinase mediated the phosphorylation of Y527. Named C-

terminal Src kinase, or Csk, this kinase is now believed to be another mechanism in which to negatively regulate the activity of c-Src (Imamoto and Soriano, 1993). In conjunction with this kinase, phosphatases such as Shp-2 (Walter et al., 1999) and PTP1A (Zheng et al., 2000) also target Y527, thereby activating the c-Src kinase. Csk null mice display an increase in Src family kinase activity and altered cytoskeletal architecture (Imamoto and Soriano, 1993), further suggesting the importance of this kinase in c-Src regulation.

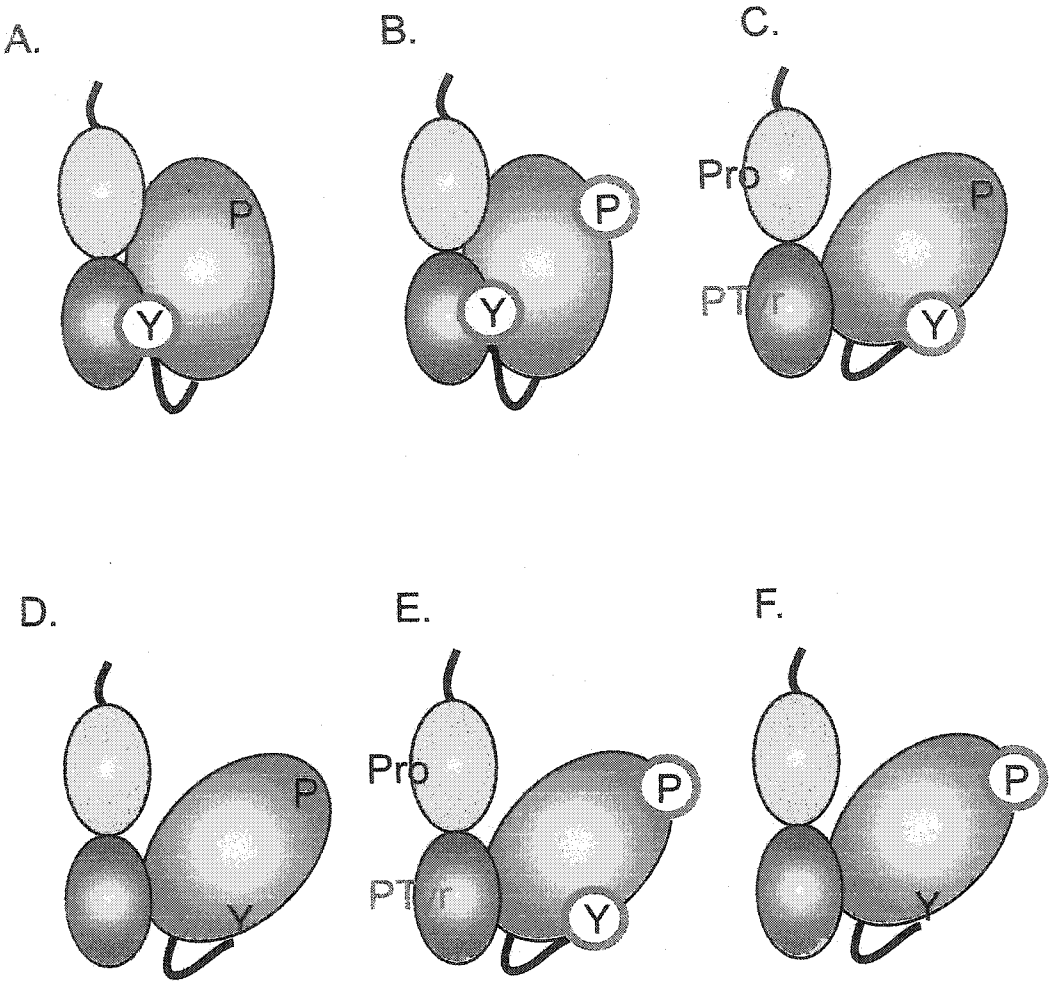
1.3.2 Regulation of c-Src activation

With the discovery of the cellular counterpart of v-*src* it was clear that the difference in kinase activity and transforming potential was due to the loss of what is now referred to as the regulatory tail. The modular nature of c-Src provided an initial hypothesis as to how the activity of this molecule is regulated. Within this region a single conserved phosphotyrosine was discovered which proved to display an affinity, albeit weak, to its own SH2 domain (Bibbins et al., 1993), thus it was hypothesized that Y527 may itself be a target for its own SH2 domain, in effect inactivating itself by creating an intramolecular association. Deletion analysis utilizing wild type c-Src as well as site directed mutagenesis specifically converting this tyrosine to phenylalanine further suggested the importance of this residue in regulating the activity of c-Src. Recent crystal structure has confirmed the hypothesis of an autoregulatory mechanism where the molecule folds upon itself in order to inactivate its catalytic domain (Figure 1.3).

The inactive conformation utilizes both its SH3 and SH2 domain to recognize specific sequences found within the linker region of the molecule as well as the regulatory tail respectively. The inactive molecule is phosphorylated at tyrosine 527 within the regulatory tail providing a docking site for its own SH2 domain. The SH3 domain recognizes a proline rich motif that resides within the linker region between the SH2 domain and the kinase domain further stabilizing the closed conformation. Recent molecular dynamic simulations confirms the importance of the linker region in locking the molecule in an inactive conformation (Young et al., 2001). Tyrosine 416, found

Figure 1.3 Models of activation of c-Src.

The c-Src PTK can adopt a number of conformations that modulate its activity. The inactive conformation utilizes both its SH3 (blue oval) and SH2 domain (purple oval) to recognize specific sequences found within the linker region of the molecule as well as the regulatory tail respectively. As depicted in (A), the inactive molecule is phosphorylated at tyrosine 527 within the regulatory tail (Y encircled red) providing a docking site for its own SH2 domain. The SH3 domain recognizes a proline rich motif that resides within the linker region between the SH2 domain and the kinase domain (not shown) further stabilizing the closed conformation. Tyrosine 416, found within the catalytic region is unphosphorylated (designated P), thereby blocking the loading of ATP into the activation pocket. (B) Even with the phosphorylation of Y416 (P encircled red) in the closed conformation, the molecule is not able to efficiently phosphorylate potential substrates. The activation of c-Src can be mediated by a number of ways. (C) For example, potential substrates may compete for the SH3 (represented by Pro) or SH2 regions (represented by Ptyr) of Src, displacing these domains from their intramolecular sites of association. However, unless Y416 is phosphorylated the kinase activity is suppressed. c-Src catalytic activity in this conformation therefore may not provide the necessary means of phosphorylating and activating its substrates. (D) Similarly, an open conformation may also be achieved by the dephosphorylation of Y527, disengaging the SH2 domain from the regulatory site. However, the highest catalytic activity is found when Y416 is phosphorylated and the molecule is in the open conformation either by substrate competition (E) , or by the dephosphorylation of Y527(F)


A.
P
Y
B.
P
Y
C.
Pro
P
PTyr
Y
D.
P
Y
E.
Pro
P
PTyr
Y
F.
P
Y

within the catalytic region is unphosphorylated, thereby blocking the loading of ATP into the activation pocket. Even with the phosphorylation of Y416 in the closed conformation, the molecule is not able to efficiently phosphorylate potential substrates.

The activation of c-Src can be mediated by a number of ways. For example, potential substrates may compete for the SH3 or SH2 regions of Src, displacing these domains from their intramolecular sites of association. Indeed, the displacement of both domains by external ligands can overcome the high level of entropic energy observed between the intramolecular association (Sicheri and Kuriyan, 1997). However, unless Y416 is phosphorylated the kinase activity and associated oncogenic potential is suppressed (Snyder and Bishop, 1984). c-Src catalytic activity in this conformation therefore may not provide the necessary means of phosphorylating and activating its substrates. Similarly, an open conformation may also be achieved by the dephosphorylation of Y527, disengaging the SH2 domain from the regulatory site. Phosphatases such as PTP1A have been implicated to play a role in the dephosphorylation of Y527 (Zheng et al., 2000). However, the highest catalytic activity is found when Y416 is phosphorylated and the molecule is in the open conformation either by substrate competition or by the dephosphorylation of Y527 reviewed in (Superti-Furga, 1995).

1.3.3 Genetic analysis of c-Src function

The Src protein kinase is found ubiquitously in mammalian tissues, and is associated with a wide variety of signaling systems. Given the universal pattern of c-Src expression, it was believed that this protein very likely played important roles in the maintenance of a number of cellular functions. Indeed, an analysis of the proteins that have been found to be altered with the introduction of v-Src has revealed a number of potential targets, many being associated with the cytoskeleton such as focal adhesion kinase (FAK), paxillin and p130Cas. Other targets include the serine/threonine kinase protein kinase Cδ (PKCδ), phospholipid kinases such as phospholipase C-γ (PLC-γ) and phosphatidylinositol 3' kinase (PI3'K), small GTP-regulatory proteins such as p120

RasGAP, phosphatases SHP-1 and PP2a and adaptors such as Shc and Dok (Thomas and Brugge, 1997). The diverse number of proteins that associate with c-Src in turn has been found to translate into a wide range of biological functions such as adhesion and spreading, focal adhesion formation and disassembly, migration, cell cycle progression, apoptosis, differentiation, and transcription. While cell culture data has shed much light on the function of c-Src, the basic role of c-Src in development was unknown. The advent of genetic ablation techniques paved the way to address the question of the role of c-Src in normal development.

To this end it was found that c-*src* null mice do survive past birth however they suffer from osteopetrosis, an osteopenic defect that manifests itself as an overall increase in bone density. The high rate of mortality and failure to thrive displayed by c-*src* null mice is partially due to the lack of incisor eruption through the jawbone attributed to an inability of osteoclasts to generate a channel for the developing incisors (Soriano et al., 1991). The osteopetrotic phenotype is not attributable to a loss of osteoclast numbers within bone, however it is attributable to the inability of osteoclasts to resorb bone, in effect altering the balance between bone deposition and bone resorbtion (Lowe et al., 1993). It appears that the ruffled border that contacts the surface of the bone is defective leading to an inability to efficiently localize hydrolases that acidify the bone surface (Boyce et al., 1992). Mechanistically, the loss of c-Src may affect the cytoskeleton, thereby altering the ability of structures such as the ruffled border to form. Indeed, the deregulation of c-Src via the loss of the negative regulator Csk, results in the alteration of cytoskeletal proteins such as paxillin, talin and cortactin (Imamoto and Soriano, 1993). Alternatively it is possible that the loss of c-Src may have altered the ability of osteoclasts to efficiently traffic hydrolase containing vesicles to the surface.

While the genetic ablation of c-*src* attempts to address the role of this gene in development, the global loss of function may alter critical signaling components that are required for other molecular systems or pathways, generating indirect effects on phenotype. To circumvent this possibility, transgenic mice that expressed a kinase defective c-*src* in osteoclasts via the promoter from the *tartrate resistant acid*

phosphatase (*TRAP*) gene were generated (Xing et al., 2001). Consistent with previous data, *TRAP-Src251* transgenic mice also display an osteopetrotic phenotype that is associated with an increase in osteoclast apoptosis and a decrease in bone resorbsion. Interestingly, the expression of a *TRAP-SrcK295M* in a c-*src* null genetic background resulted in the partial rescue of the osteopetrotic phenotype in mice (Schwartzberg et al., 1997). This observation suggests that within osteoclasts, c-Src might play a role in osteoclast survival that is not dependent on its catalytic activity. Consistent with this observation, c-*src* null fibroblasts which display a defect in cell spreading in the presence of fibronectin can be rescued not only with wild-type c-Src but also with a truncation mutant of c-Src (Src251) that lacks the kinase and regulatory region of the protein (Kaplan et al., 1995). Taken together, this implies that the catalytic activity of c-Src may generate an anti-apoptotic signal required for the survival of osteoclasts. Simplistically, it appears that c-Src can alter the function of osteoclasts in a kinase independent as well as a kinase dependent manner, the latter possibly involving an apoptotic pathway that insures osteoclast survival. The overexpression of Src251 may also inhibit the function of other molecules required for the regulation of osteoclasts, thereby inducing apoptosis.

Recently, the molecule osteoprotegrin ligand (OPGL) was discovered to play a key role in osteoclastogenesis. In the presence of CSF-1, OPGL can induce osteoclastogenesis as well as osteoclast activation in the presence of osteopenic factors such as vitamin D, interleukin-11, prostaglandin E_2 and parathyroid hormone. The genetic ablation of OPGL has led to the discovery that this factor is absolutely required for normal bone development (Kong et al., 1999). Null mice are found to be severely osteopetrotic with shortened long bones, higher bone density with the almost complete occlusion of the marrow spaces of the epiphysis, metaphysis and diaphysis, failure of incisor eruption, and no TRAP positive staining of osteoclasts. Interestingly, *TRAP-Src251* mice display a defect in osteoclast survival in the presence of OPGL suggesting that this ligand functions through c-Src (Xing et al., 2001). Indeed, c-*src* null osteoclasts also respond poorly to OPGL mediated survival, supporting the hypothesis that osterclastogenesis mediated by OPGL may function through c-Src.

1.3.4 Interactions between receptor tyrosine kinases and c-Src

The c-Src protein tyrosine kinase (PTK) has been implicated to be an important mediator of receptor signaling in a variety of systems. Indeed, the mutation of Y579 or Y581 on the platelet derived growth factor receptor (PDGF-R) leads to a reduction in c-Src activity and its association to the receptor. In addition, mutation of both c-Src binding sites on the PDGFR is found to ablate the kinase activity of the receptor (Mori et al., 1993). PDGF has also been found to influence the cell cycle via c-Src since microinjection of dominant negative forms of c-Src inhibits PDGF mediated entry into S phase (Twamley-Stein et al., 1993). Interestingly, a gene independent of the endogenous c-*src* gene was identified to contain both SH3 and SH2 domains that are highly similar to the domains found within c-Src but is devoid of catalytic activity. The discovery led to the hypothesis that a naturally occurring dominant inhibiting protein similar to c-Src can antagonize receptor signaling. Indeed, the SH2 domain of this Src-like adapter protein (Slap) (Roche et al., 1998) was found to have a similar affinity to Y579 and Y581 on the PDGF receptor. Slap is myristylated, colocalizes with c-Src and was found to negatively regulate mitogenesis and PDGF mediated DNA synthesis in fibroblasts both in a competitive fashion via its SH2 domain.

PDGF receptor function is also dependent on the presence of other proteins found within the receptor complex; these proteins in turn appear to be dependent on the presence of c-Src. For example, STAT3 (Wang et al., 2000), Shc, c-Cbl and protein kinase C delta (Blake et al., 2000) all appear to be c-Src substrates in the presence of PDGF. Although the evidence suggests an importance for c-Src in PDGF receptor signaling, triple null Src, Yes and Fyn (SYF) embryonic fibroblasts are however, competent in PDGF mediated signal transduction. What does seem to be impaired in these SYF cells is the ability to activate integrin mediated cell signaling. This data suggests that while the Src family may promote functions associated with cellular architecture mediated by PDGFR, cycle progression beyond S phase is not dependent on the ability of the Src family to associate with the receptor (Klinghoffer et al., 1999). This

evidence appears to be counter to the previous mutagenesis data, however recently it was suspected that the technique utilized to generate the immortal cell lines might have led to the ability to activate pathways downstream of the receptor independent of c-Src (Broome and Courtneidge, 2000).

Within another receptor system, the activation of the colony stimulating factor-1 receptor (CSF-1R) with CSF-1 appears to be also dependent on the presence of c-Src. Indeed, the mutation of the c-Src binding site on the colony stimulating factor-1 receptor (CSF-1R) dramatically decreases the kinase activity of c-Src (Alonso et al., 1995). Furthermore, the inability of a mutant CSF-1R to activate MAPK, cyclinD1 and c-Myc can be rescued with the overexpression of v-Src (Aziz et al., 1999). Dominant negative c-Src microinjected into fibroblasts blocks CSF-1 mediated DNA synthesis and entry into S phase (Roche et al., 1995b), suggesting that c-Src mediates an important signaling role immediately downstream of the CSF-1R. Additional examples of dominant negative forms of c-Src that inhibit ligand induced signaling from RTK's includes the epidermal growth factor receptor (EGFR) and the nerve growth factor receptor (Kremer et al., 1991; Roche et al., 1995b).

1.4 Reproductive development

1.4.1 Nuclear receptor family

The nuclear receptor family comprises of six distinct classes that in total account for 26 individual nuclear receptors (Table 1.1). In common between these receptors are their structures and associated functions of which have been divided into six domains defined by three general regions: the NH2-terminal A/B activation domain, the DNA binding C domain, and the COOH-terminal D/E/F ligand-binding domain (Figure 1.4).

In transactivation studies using reporter assays, the A/B region has the ability to activate transcription independent of ligand association. Looking specifically at the estrogen receptor, this activation function (AF-1) within the B domain is considered constitutive and autonomous. Mutation analysis shows that autonomous AF-1 function is

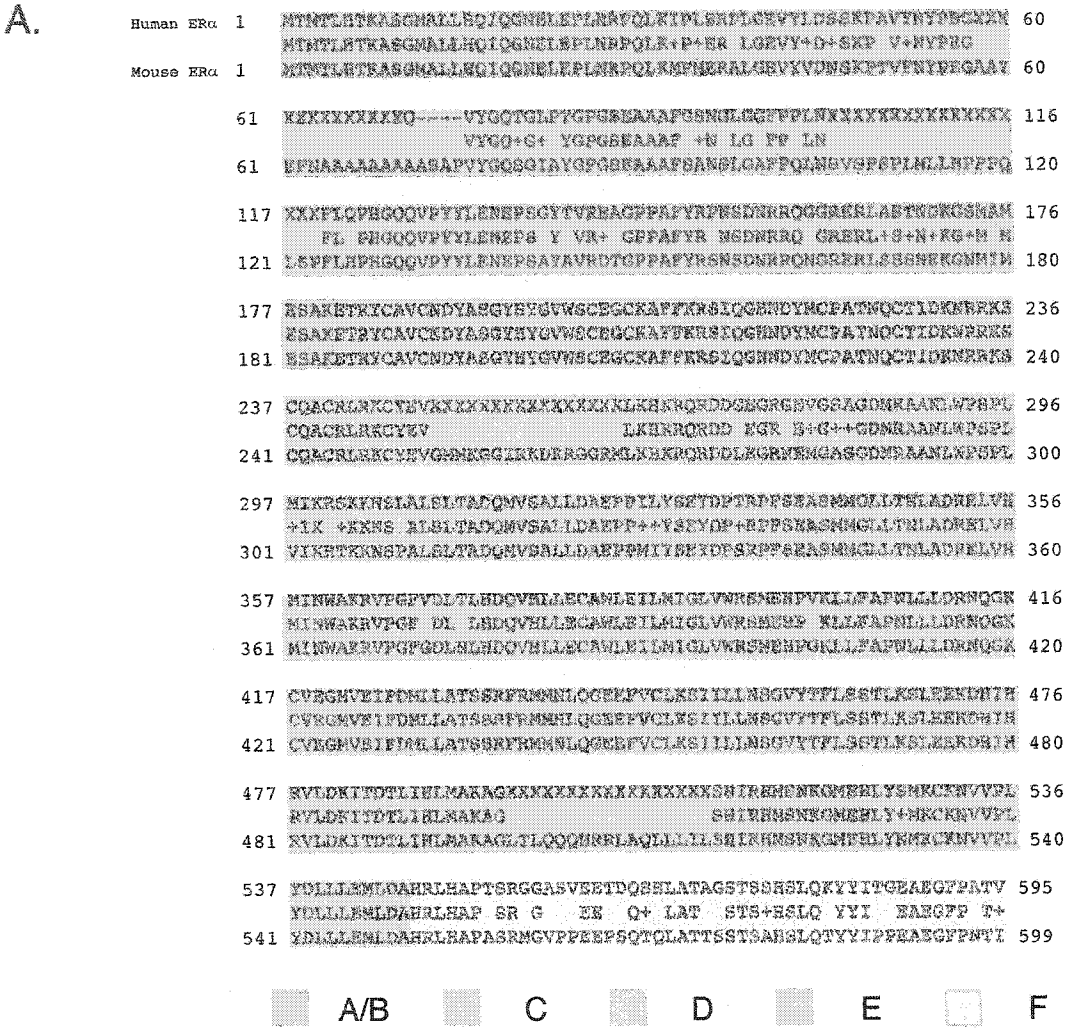
Table 1.1 Nuclear Receptor Superfamily

	Receptor/ subtypes	Name	Ligand	Response element
Class 1	TR α β	Thyroid hormone receptor	Thyroid hormone (T_3)	Pal, DR-4, IP
	RAR α β γ	Retinoic acid receptor	Retinoic acid	DR-2, DR-5 Pal,
	VDR	Vitamin D receptor	1-25$(OH)_2$ vitamin D3	IP
	PPAR α β γ	Peroxixome proliferator activated recepor	Benotriene B4; Wy 14.643	DR-3, IP-9
			Eicosaniods; thiazolidinediones (TZDs); 15-deoxy-12,41-prostaglandin J_2; polyunsaturated fatty acids	DR-1
	PXR			
	CAR/MB67 α β	Pregnane X receptor	Pregnanes; C21 steroids	DR-3
		Constitutive androstane recetor	Androstanes; 1-4-bis[2-(3,5-dichloropyridyloxy)}benzene	DR-5
	LXR α β	Liver X receptor	Oxysterols	DR-4
	FXR	Farnesoid X receptor	Bile acids	DR-4, IR-1
	RevErb α β	Reverse ErbA	Unknown	DR-2, Hemisite
	RZR/ROR α β γ	Retinoid Z receptor/retinoic acid-related orphan receptor	Unknown	Hemisite
	UR	Uniquitous receptor	Unknown	DR-4
Class II	RXR α β γ	Retinoid X receptor	9-*Cis*-retinoic acid	Pal, DR-1
	COUP-TF α β γ	Chicken ovalbumin upsream promotor transcription factor	Unknown	Pal, DR-5
	HNF-4 α β γ	Hepatocyte nuclear factor 4	Fatty acyl-CoA thioesters	DR-1, DR-2
	TLX	Tailles-related receptor	Unknown	DR-1, Hemisite
	PNR	Photoreceptor-specific nuclear receptor	Unknown	DR-1, Hemisite
	TR2 α β	Testis receptor	Unknown	DR-1 to DR-5
Class III	GR	Glucocorticoid receptor	Glucocorticoids	Pal
	AR	Androgen receptor	Androgen	Pal
	PR	Progesterone receptor	Progesterone	Pal
	ER α β	Estrogen receptor	Estradiol	Pal
	ERR α β	Estrogen-related receptor	Unknown	Pal, Hemisite
Class IV	NGFI-B α β γ	NGF-induced clone B	Unknown	Pal, DR-5
Class V	SF-1/FTZ-F1 α β	Steroidogenic factor 1/Fushi Tarazu factor 1	Oxysterols	Hemisite
Class VI	GCNF	Germ cell nuclear factor	Unknown	DR-0
Class 0	SHP	Small heterodimeric partner		
	DAX-1	Dosage-sensitive sex reversal		

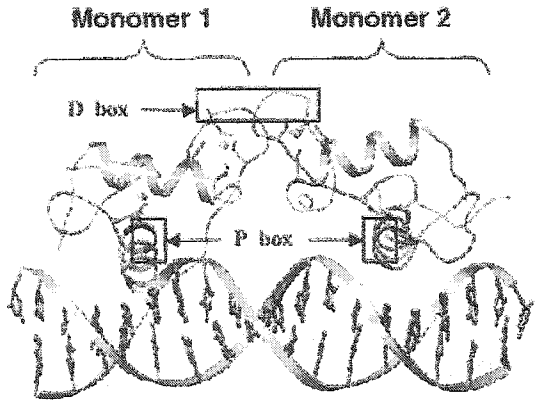
Figure 1.4 Amino acid sequence of estrogen receptor α.

(A) Shown is an alignment of human and mouse estrogen receptorα. Also shown are the major domains indicated as A/B (NH_2-terminal region that contains the ligand-independent AF-1 transactivation domain); C (conserved DNA binding domain); D (variable linker region); E (Ligand-binding and dimerization domain that contains the ligand-dependent AF-2 region) and F (COOH-terminal region). (B) Three-dimensional X-ray structure of ERα associating with DNA. Shown are two monomers joined at the dimer interface via the 'D box' while associating with the palindromic response element found in large groove of the DNA helix via the 'P box' (Figure from Ruff et al., 2000).

A.



B.



absolutely required in its ability to transactivate (Berry et al., 1990). *In vitro* studies that try to define the biological consequence of the AF-1 region have shown that transactivation of Gal4 responsive promoter elements are efficiently mediated by AF-1/Gal4 chimeric receptors in chicken embryonic fibroblasts but not in the human cell line HeLa (Berry et al., 1990; Tora et al., 1989). This specificity may reside in the ability of the AF-1 region to associate with distinct proteins found within individual cell types.

The highest degree of conservation found in the nuclear hormone receptor family is within the C region, this region having been found to mediate multiple functions such as protein-DNA interactions via the DNA binding domain (DBD), protein-protein interactions, dimer formation, and nuclear localization. The solution structure of the ER reveals 2 separate zinc fingers motifs that can mediate receptor dimerization and DNA association (Schwabe et al., 1993). The first zinc finger juxtaposes itself with the large groove of the DNA helix, bringing the 'P-box' in contact with key base pairs found within the spacer region flanked by the palindromic estrogen response element (ERE) sequence, thereby generating a mechanism of specificity at the level of DNA (Ruff et al., 2000; Umesono and Evans, 1989). The second zinc finger motif harbors the 'D-box' that mediates dimer formation when both monomers are associated with DNA in a head-to-head fashion (Ruff et al., 2000). As an unliganded monomer, the receptor is believed to be complexed with proteins such as heat shock protein 90 that may regulate its activity and conformation (Chambraud et al., 1990). Also within this region, three ligand independent nuclear localization sequences are found to direct translocation into the nucleus (Ylikomi et al., 1992).

Of the COOH-terminal region that comprises D/E/F, region E is the best characterized. It is here that the hormone binding domain (HBD) and the second activation function (AF-2) region resides. AF-2 unlike AF-1 is ligand dependent in its ability to transactivate gene expression (Webster et al., 1988). Also found within this region are heat shock protein 90 binding sites, and a ligand dependent NLS (Ylikomi et al., 1992). Activation due to ligand association to this region induces a conformational shift that facilitates the association of nuclear coactivators to the receptor. The

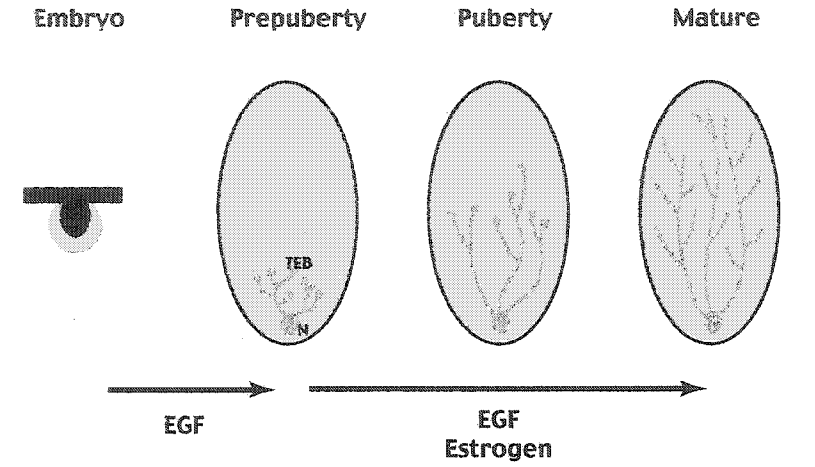
recognition motif found on coactivator proteins that associate with the estrogen receptor conform to the sequence LxxLL, this hydrophobic motif docking to the hydrophobic groove bracketed by helicies H3, H4, H5, and H12 on the receptor. While estrogen or its nonsteroidal yet similar agonist diethylstilbesterol (DES) is completely buried within the hydrophobic pocket, tamoxifen when bound to the LBD alters the conformation of H12, thereby changing the ability of this region to associate with specific coactivators required to facilitate transactivation (Shiau et al., 1998). This underscores the fundamental mechanism of how all antiestrogens are competitive inhibitors for the LBD.

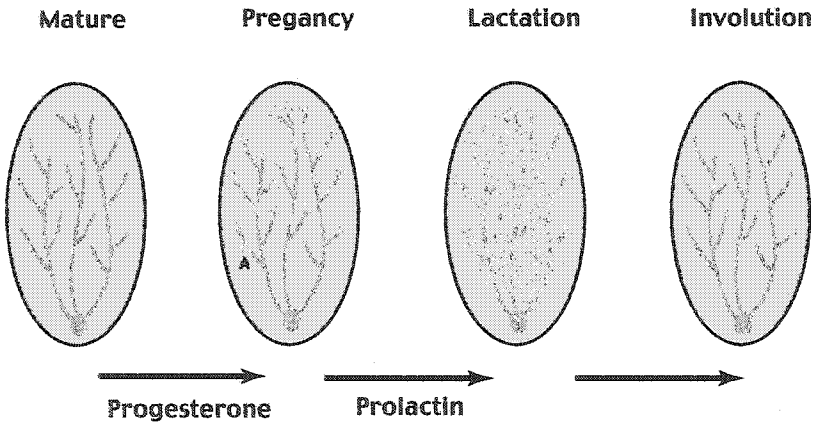
1.4.2 Early mammary gland development

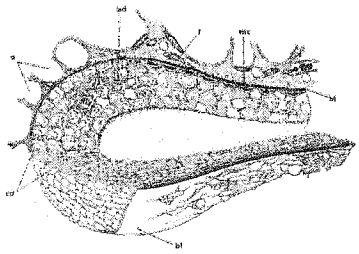
Mammary gland development (Figure 1.5) begins with the formation of two pairs of five ectodermal placodes at embryonic day 10-11 (E10-11), which run ventrally on either side of the embryo. While the development of the mammary buds at this stage is autonomous in that no systemic signals are required for their developmental coordination, it is dependent on cell specific signals that are derived from the interactions between the mammary mesenchyme and the mammary epithelial bud. At E12-14 the mammary mesenchyme forms, surrounds and envelops the epithelial bud precursor, committing this structure to form the mammary epithelial tree. As the epithelial bud proceeds in its development the fat pad precursor develops posterior to the epithelial bud at E16. Significantly, the independent development of the fat pad from mesenchymal fibroblastic cells promotes the proper branching architecture found in a typical mammary tree (Hennighausen and Robinson, 2001). The absence of the fat pad mesenchyme results in an abnormally compact branching phenotype, emphasizing the importance of the stromal-epithelial cell relationship in the proper development of the mammary gland. The molecular signals that coordinate the stromal-epithelial interactions that generate the mammary tree reside in both the mammary epithelial bud and the mammary mesenchyme. During the embryonic and prepubertal stages of development the transcription factors Lef-1 (Satokata et al., 2000; van Genderen et al., 1994), Msx-1 and

Figure 1.5 Mammary gland development.

Shown are the major developmental stages of mammary gland development in the mouse. Embryonic structures are shown as an epithelial bud (black) surrounded by stroma (grey). Postnatal development is shown as a fat pad (blue; stroma) with the growing ductal architecture lead by terminal end buds (TEB) developing from the nipple (N). Epithelial ducts are shown as solid lines (D) while lobuloalveolar structures as yellow circles (A). The major hormones are shown at each stage of development. Figure adapded from (Hennighausen and Robinson, 2001) and (Daniel, 1987).


Embryo
Prepuberty
Puberty
Mature
TEB
N
EGF
EGF
Estrogen


Mature
Pregancy
Lactation
Involution
A
Progesterone
Prolactin



Msx-2 and the growth factor BMP4 appear within the mammary mesenchyme (Phippard et al., 1996) however their roles are not well characterized.

During the 4th to 6th postnatal week the mammary tree begins to ramify into the mammary fat pad. At this stage the morphology of the mammary gland is predominantly ductal with minimal side branching and terminal end buds (TEBs) being clearly present. The TEB consists of a border of non-differentiated cap cells that form the outermost layer followed by 3 to 4 cell layers of what will eventually form the single layer of luminal epithelial cells. In addition to ductal epithelial cells, other cells found distal to the TEB are fated to form myoepithelial cells that surround each duct. The highest level of mitosis and cellular migration is thus found to occur in the TEBs. In contrast, the ductal epithelial cells derived from the leading end bud remain quiescent upon differentiation (Hennighausen and Robinson, 1998).

1.4.3 Genes implicated in reproductive function

Reproductive development involves the complex coordination of multiple cues derived from both endocrine and cell-to-cell signals. The cellular translation of these signals result in the generation of specific biological outcomes driven by a multitude of intracellular signaling cascades. The regulation of alveolar formation and ductal branching during the development of the mammary gland depends on the presence of a number of ovarian hormones, of which 17β-estradiol is the most predominant and to date, the best characterized. Generally the molecular mechanism of steroid induced signal transduction is poorly understood, however stimulation with estrogen can induce the activation or modification of a number of cytoplasmic signaling molecules such as Ras, MAPK, Shc, the signal transducers and activators of transcription (STAT) family, and cyclinD1.

1.4.3a *Estrogen receptor*

Of all the signals that regulate reproductive development, estrogen and its receptor has been the best characterized to date. The generation of the estrogen receptor α

knockout (αERKO) mouse has provided great insight into the biological importance of the ER in reproductive function. Indeed, the defects that have been uncovered not only include the reproductive organs, but also skeletal and cardiovascular defects (Curtis Hewitt et al., 2000). While the loss of the estrogen receptor negatively affects the development of structures such as the mammary gland, the various phenotypes may also be due to defective estrogen signaling via the hypothalamic/pituitary axis (Bocchinfuso et al., 2000). This underscores the fact that reproductive and hormonal regulation is controlled both by a neuronal component and by immediate systemic factors, generating both complexity in phenotype and difficulty in interpretation.

Mammary gland development: The development of the mammary gland is exquisitely sensitive to hormone levels during early development and onward in a cyclical fashion. While prepubertal ductal outgrowth generally keeps pace with the development of the mouse, upon puberty the ducts rapidly develop into the fat pad stroma, its' architecture being determined by the leading terminal end-buds. This stage of mammary gland development appears to be predominantly driven by estrogen and EGF, and ceases when the terminal end-bud reaches the end of the fat-pad where it then goes into a quiescent state. Early studies that utilized ovariectomized mice demonstrated the importance of ovarian factors in ductal development. Indeed, the ductal regression observed in the ovariectomized mice can be rescued with the addition of exogenous estrogen (Mixner, 1942). Similarly, the mammary glands of the ERKOα mouse leads to a dramatic loss of ductal outgrowth, with only vestigial ducts appearing at the nipples suggesting that the estrogen receptor is required for the proper development of the mammary gland (Korach et al., 1996). However, it appears that mammary gland development is far more complex, in that stromal-epithelial communication as well as the involvement of the hypothalamic-pituitary axis, both play roles in development. Early data suggested the involvement of an epithelial-stromal component in the proper development of the mammary gland using tissue recombinants (Cunha, 1994). These studies demonstrate that the estrogen receptor is critical for mammary gland development (Cunha et al., 1997). The mammary gland defect in ERKOα mice can be rescued with the

introduction of a wild-type pituitary gland within the kidney capsules suggesting that the mammary gland defect in the ERKOα mice is also due to defective hypothalamic-pituitary axis function (Bocchinfuso et al., 2000).

Ovarian development: The ovaries of the αERKO mouse prior to puberty appear normal, however it is clear that a defect does exist that manifests itself upon sexual maturity and the commencement of follicular development. In fact, the follicles display an anovulatory phenotype that can be grossly described as hemorrhagic and cystic. Indeed, female αERKO mice are infertile and possess hyperemic ovaries that harbor primary and secondary follicles that do not progress to the antral stage. Instead, large atretic haemorrhagic cysts develop suggesting that a block in folliculogenesis is occurring prior to the antrum stage (Korach et al., 1996; Lubahn et al., 1993). Consistent with the above evidence for the importance of the ER in ovarian development, P450 aromatase knockout mice that lack estradiol but harbor high levels of LH and FSH, also display an anovulatory phenotype (Fisher et al., 1998). Although the paucity in development is obvious, the molecular mechanism as to how the ovarian defect occurs is unclear. For example, while the genetic loss of the estrogen receptor is associated with high levels of LH and FSH (Schomberg et al., 1999), molecular confirmation of both their roles in contributing to an ovarian phenotype appears complex. Both the transgenic overexpression of LHβ, and long-term treatment with antiestrogens generate a hyperemic ovarian phenotype similar to the ERKO mouse which is consistent with the possible importance of LH and ERα in ovarian development (Risma et al., 1995; Sourla et al., 1997). The genetic ablation of FSHβ, which otherwise has abundant levels of LH, however does not display an ovarian phenotype similar to the αERKO mouse (Kumar et al., 1997). These lines of evidence only suggest that other secondary, interacting effects, such as the presence of FSH, are most likely at work to generate the ovarian defect. Since successful follicle maturation is dependent on the synchronization of both FSH and LH via estradiol, it has been hypothesized that the high levels of LH may upset follicular maturation and instead initiate premature luteinization or atresia, possibly via an apoptotic pathway (Billig et al., 1993). Given this explanation, it does not shed light on

the molecular mechanism involved in ovarian development as seen in the ERKO, let alone the normal physiological setting.

1.4.3b *c-Src kinase in reproductive development*

While genomic events are classically associated with the activation of the ER, non-genomic events are also an important mechanism in estrogen mediated signal transduction. For example, stimulation of MCF-7 cells with estrogen correlates with an increase in a number of tyrosine phosphorylated proteins, notably within the 60kDa range. Subsequent analysis revealed that these proteins represent the Src family kinases (Migliaccio et al., 1993). Indeed, the increase in c-Src tyrosine phosphorylation also correlated with an increase in its kinase activity. In addition, specific c-Src inhibitors can block estrogen mediated ER signaling (Migliaccio et al., 1996). Furthermore, the phosphorylation of tyrosine 537 on ERα by c-Src is critical for receptor dimerization and activation of itself as well as the Ras/MAPK pathways and subsequent binding of the ER to the estrogen response element (Arnold et al., 1995). Interestingly, tyrosine 537 has been found to suffer somatic mutations in breast cancer cells derived from metastatic tumors that are resistant to anti-estrogens suggesting that this critical tyrosine residue is selected against in order to progress to an aggressive tumor phenotype (Zhang et al., 1997). With the significance of the ER in mammary gland development established as demonstrated by the ERα knockout (ERKO) mouse in conjunction with the importance of c-Src in ER signaling, the above data suggests strongly that c-Src may play an important role in ERα mediated mammary gland development. However, biological data that correlates a role for c-Src in ERα induced mammary gland development *in vivo* is lacking.

1.4.3c *Signal transducers and activators of transcription (STATs)*

First identified as mediators of interferon induced signaling, the STAT proteins are now known to be activated by a large number of ligands. Depending on the specific ligand, different combinations of dimers form from the more than seven STAT factors

now characterized. For example, interferon (IFN) α stimulation results in the phosphorylation of specific and conserved residues such as tyrosine 701 on STAT1, potentiating activation via dimerization with either itself or as a STAT1:STAT2 heterodimer. This results in the translocation of the STAT complex into the nucleus where it associates with the IFN-stimulated response element (ISRE) found upstream of IFNα responsive genes. Alternatively, IFNγ results in the exclusive homodimerization of STAT1, its translocation into the nucleus and association with IFNγ-activated sites (GAS-sequences) in the promoters of IFNγ responsive genes. As alluded to above, other ligands have been found to activate STAT1 such as EGF and PDGF again resulting in the translocation of the STAT complex to the nucleus. Here, it associates with transcriptional elements such as ones found adjacent to the serum response element (SRE) of c-*fos*, the sis-inducible element (SIE) (Bromberg and Darnell, 2000).

A number of STAT family members have been found to play a role in the developing and mature mammary gland both in cell culture and in animal model systems. For example, the lactogenic factor progestin has been found to activate the translocation of STAT5 to the nucleus, potentially associated with the progesterone receptor (Richer et al., 1998). The genetic ablation of STAT5 results in a lactation defect in mice. Genetic evidence appears consistent with the role of STAT5 in prolactin receptor function (Liu et al., 1997), given that the loss of the progesterone receptor also leads to lactation defects (Gallego et al., 2001). While STAT5 appears to play a role in the reproductive stages of pregnancy and lactation, STAT3 activation appears to correlate with involution given its high levels of expression at this stage (Li et al., 1997). The role of STAT3 within the mammary gland however is unclear due to its lethality upon the genetic deletion of this gene (Takeda et al., 1997). To address the significance of STAT3 in the mammary gland a Cre-lox approach was taken. To this end it, was found that the specific deletion of STAT3 within the mammary gland resulted in an involution delay that correlated with a decrease in epithelial apoptosis (Chapman et al., 2000; Chapman et al., 1999). While STAT5 activation is influenced by a known factor that influences lactation, prolactin, activation of STAT3 does not appear to be influenced by mammopoetic factors.

However, it is influenced by EGF, a known factor that has been implicated to cooperate with estrogen (Gallego et al., 2001). Given that other cytoplasmic factors such as c-Src have been found to activate STAT3, it will be interesting to see if c-Src can influence STAT3 function in mammary gland development.

1.4.3d *CyclinD1*

From a quiescent state, stimulation with estrogen has been shown to drive cells into the G1 phase of the cell cycle (Leung, 1987). In contrast, treatment of ER positive cells with antiestrogens correlates with a decrease in thymidine incorporation and arrest in G1 while ER negative cells do not display a decrease in DNA synthesis when in the presence of antiestrogens (Prall et al., 1997). Further characterization of the early events after stimulation with estrogen revealed a role for cyclins in estrogen mediated cell cycle progression. Indeed, antiestrogens can reduce cyclinD1 mRNA levels in addition to cyclinD1 protein levels (Prall et al., 1997). Conversely, estradiol stimulation results in an increase in cyclinD1 levels as well as an associated increase in cdk activity (Musgrove et al., 1996). Recent evidence has revealed that cyclinD1 may not only play a role in cell cycle progression, but also play a role in mediating nuclear complexes driven by estrogen in that cyclin D1 harbors a LxxLL motif that has been found to associate with estrogen receptor complexes. Indeed, cyclinD1 has been found to associate with the receptor itself as well as transactivators such as SRC-1 (Zwijsen et al., 1998). The discovery of cyclinD1 as a mediator of mammary gland development was a serendipitous observation upon generating the knockout mouse. The mammary glands of these mice develop normally, however the defect presents itself upon lactation and the initiation of regression again suggesting a role for this protein in mammary gland function (Fantl et al., 1995).

1.5 Prognostic indicators in breast cancer: c-ErbB2 and the estrogen receptor

While the overexpression of c-ErbB2 has been found to efficiently initiate transformation in cell culture and animal models, the role of this receptor as a prognostic indicator in human cancers is not as clear. What is known is that its overexpression is

found in 30% of human breast cancers of which 70% of these are due to the amplification of the gene (de Cremoux et al., 1999). Univariate analysis has shown that the overexpression of c-ErbB2 correlates with a more aggressive tumor phenotype, antiestrogen resistance, higher cellular proliferation, poor histological and nuclear grade and a general negative prognosis. However, given these observations it becomes increasingly unclear as to the value of c-ErbB2 as a prognostic indicator in multivariate analysis due to the association of c-ErbB2 with other strong indicators of prognosis (reviewed in (Clarke et al., 2001).

The initial studies that suggested a role for ovarian hormones in breast cancer was realized in 1896 when Beaston noted that oophorectomy correlated with the regression of advanced breast cancers (Beatson, 1896). Four years later, the same procedure resulted in the regression of one-third of all clinical cases of women with advanced breast cancer (Boyd, 1900). While this represented the first clinical evidence for estrogens in breast cancer, the mechanism of estrogen action was poorly understood. With the rapid elucidation of the basic mechanism of estrogen action, the estrogen receptor has now become the most successful therapeutic target for breast cancer. Indeed, with more than 100 million patient hours of clinical experience, anti-estrogens are the mainstay of current adjuvant therapy for breast cancer, however little is known as to the regulation of estrogen receptor signaling and is plagued by seemingly contradictory data. For example, studies have shown that women who have a normal reproductive lifespan display a higher incidence of breast cancer when compared to women who reach early menopause under natural circumstances suggesting that the loss of estrogen provides a protective or preventative effect (Hulka and Stark, 1995). However, estrogen receptor negative women who suffer from breast cancer develop tumors that are highly aggressive suggesting that the deregulation of the estrogen response can exacerbate the tumor phenotype. Most likely these differences are due to the fact that one is in the normal context prior to disease onset while the latter is with an established tumor load.

It is well recognized that both the EGFR family and the estrogen receptor are strong prognostic indicators of human breast cancer. Indeed, both receptor systems have

been found to be intimately associated in regulating the cellular functions in both normal and neoplastic cells. Given this clinical association, the biochemical mechanism of how these two receptors regulate cellular function in transformed cells is complex. For example, studies have suggested that the overexpression of c-ErbB2 correlate negatively with estrogen receptor levels (Newman et al., 2000), to this end engendering a more aggressive tumor phenotype and a resistance to antiestrogen treatment. However, not all c-ErbB2 overexpressing tumors that display a high degree of aggressiveness have low levels of estrogen receptor. Furthermore, while it is known that estrogen can downregulate c-ErbB2 transcription, tamoxifen (TAM) has been found to increase c-ErbB2 levels (Antoniotti et al., 1992) yet still provide a protective role in breast cancer. How is it that tumors expressing high levels of c-ErbB2 still fall under the regulation of the estrogen receptor?

The classical estrogen receptor pathway involves receptor activation subsequent to ligand binding, resulting in receptor dimerization within the nucleus. Upon dimerization, it can then associate with estrogen response element (ERE) containing genes to transmodulate gene transcription. Alternatively, a ligand independent pathway has also been speculated whereby kinases can modify the receptor and thus its localization and function. In addition, ERE independent mechanisms exist that can drive transcription from alternate genetic elements upstream of specific genes such as sp1 AP-1 and RXR elements (MacGregor and Jordan, 1998). Finally, a cytoplasmic estrogen receptor fraction appears to play a role in initiating signal transduction, for example the c-Src, the Ras/MAPK pathway as well as the PI3'K pathway all appear to associate or impinge on estrogen function (Castoria et al., 2001; Migliaccio et al., 1996; Migliaccio et al., 1998). Thus, the estrogen receptor appears to have multiple mechanisms to activate and modulate transcription and signaling pathways. In light of the multiplicity of estrogen receptor function, this may provide an explanation as to why the loss of estrogen receptor function may have a high impact on cellular function and in turn physiological consequences, for example with the treatment with tamoxifen. Moreover, the premature menopause seen by some women may negatively impact on the ability of gain- or loss-of-

function events that may occur in the normal breast to potentially develop into breast cancer.

The functional multiplicity of the estrogen receptor can also be considered counterproductive to the patient who has an established tumor load. For example, the presence of the estrogen receptor can synergize with both gain- and loss-of-function mutations already present in an established tumor thereby exacerbating the disease state. Given the importance of the estrogen receptor in reproductive tissues as well as the multiple actions of the estrogen receptor in regulating transcription, this may explain the dramatic effects of anti-estrogens in tumors despite the presence of other dominant-activating events. However, it is also well known that tumors that are classified as highly aggressive also correlate with an estrogen receptor-negative phenotype, suggesting that the loss of this receptor can exacerbate the pathology. This further implies that the ER may negatively regulate cellular proliferation. However, does the definition of an ER-negative phenotype imply a physical loss of the receptor or its function as a classical receptor? If there is no physical loss of the estrogen receptor as suggested by the definition of an ER-negative phenotype, this implies that the unresponsiveness of the receptor to compounds such as tamoxifen is due to events that provide independence from the classical competitive nature of anti-estrogens and at the same time possibly preserving its capacity to signal and even transactivate transcription.

1.6 Summary of intent

Many studies that include cell culture, animal models, and human cancers suggest that molecular events that either hyperstimulate or attenuate specific pathways can lead to the transformation and progression of normal cells to an aggressive tumor phenotype. In combination with hormonal factors, cells that are responsive to these extracellular cues can acquire a great growth advantage leading to a poor patient prognosis. A number of molecular molecules have been extensively characterized that have been associated with transformation in both animal models and human breast cancers. For example, the overexpression of the c-ErbB2/Neu receptor has been found to correlate inversely with a

positive prognosis of the patient. Closely associated with c-ErbB2, the expression of the estrogen receptor has been found to influence heavily the growth of tumors within the breast. Indeed, high levels of c-ErbB2/Neu have been found to correlate with the suppression of estrogen receptor levels, this potentially participating in the establishment of an anti-estrogen resistant phenotype observed in many patients. Evidence now exists that suggest that the c-Src protein kinase can interact and modulate the function of both the c-ErbB2 and the estrogen receptor in transformed mammary epithelial cells. The significance of this regulation is unclear. In chapter 3, I present further evidence that supports the observation that c-Src will preferentially associate with the c-ErbB2 receptor when compared to the EGFR. Furthermore, this association is not mediated by the carboxyl terminal tail like so many other molecules that fall within this class of receptor. Instead, the site of association appears to lie within the kinase region of the receptor. Interestingly, the direct association of c-Src to the receptor is observed at the site known as the activation loop, mutation of this loop ablates the transforming ability of the receptor, as does a dominant inhibiting form of c-Src. This subtle difference in substrate association is found to generate differences in MAPK activation, potentially translating into differences in biological output. Taken together, these data suggests that the association between c-Src and the c-ErbB2/Neu receptor is important in the establishment of the full catalytic activity of the receptor as well as playing an important role in Neu mediated transformation.

In chapter 4, I present evidence that the loss of c-Src can influence the development of reproductive organs, possibly due to the inability to signal from the estrogen receptor. It appears that a general delay in development is observed in female mice that are null for c-Src as assessed in the mammary gland, the ovaries and uterine tissues. Levels of estrogen receptor are down in mammary gland explants, and the expression of a dominant negative c-Src can suppress estrogen receptor levels as well as estrogen mediated translation. Similar to the c-ErbB2 receptor the presence, or absence, of c-Src appears to affect MAPK activity and in addition, levels of cyclinD1, FAK, GSK3β, STATs and Akt.

With these studies I have demonstrated the importance of c-Src when in association with two receptor families that have been heavily implicated in reproductive development and pathology, the estrogen receptor and the EGFR family member Neu/c-ErbB2. In both cases, c-Src appears to play a critical role in receptor activation as well as coordinating downstream events that impinge on reproductive development as is the case with the estrogen receptor, or in mammary gland transformation induced by Neu/c-ErbB2.

CHAPTER 2

Materials and Methods

2.1 Expression Vectors, cDNA Constructs and PCR Mutagenesis

2.1.1 Expression Vectors

The pSV2gpt expression vectors (Di Fiore et al., 1992; Di Fiore et al., 1987a; Di Fiore et al., 1987b; Di Fiore et al., 1990) harbored the EGFR, TMTK, TK1 and RT cDNA's. The TK2 and RasV12 cDNA, NeuNT and all mutants derived from NeuNT utilized the pJ4Ω expression vector. The Src251 and ShcHA cDNAs were placed into the vector pBPΩ (constructed by R. Chan) which harbors an in cis puromycin selectable marker. All the above expression vectors are driven by the Moloney murine leukemia virus long terminal repeat (MoMLV LTR). The c-SrcSH2 sequence was subcloned as a *Hind*III-*Eco*RI fragment into the GSTag expression vector, its construction was described previously (Muthuswamy and Muller, 1995). The puromycin resistance gene is driven by the phosphoglyceraldehyde kinase (PGK) promoter.

2.1.2 cDNA constructs

The NeuNT (V664E) cDNA was provided by Dr. R. Weinberg. The wild-type EGFR, TMTK, TK, TK1 and RT cDNA constructs were from Dr. P.P. DiFiore. The Src251 cDNA was provided by Dr. P. Schwartzberg. The c-SrcSH2 fusion protein was provided by Dr. B. Margolis. The RasV12 cDNA was provided by Dr. J. Massague. The PGK-puromycin construct was provided by Dr. M.A. Rudnicki.

2.1.3 PCR mutagenesis

All single-site tyrosine-to-phenylalanine mutations were generated in the context of the NeuNT cDNA. All numbering that relate to amino acid positions are from herein derived from the Neu protein sequence. In this specific version of NeuNT, a *Nco*I site

located in the 5' untranslated region of the cDNA was destroyed (engineered by D.L. Dankort). In addition, an *Eco*RI site at nucleic acid position 3800 immediately 3' to the stop codon of the NeuNT cDNA was generated. The generation of the point mutations was facilitated by subcloning a *Hin*dIII-*Nco*I fragment (nucleotides 1 to 3030, designated as pNT-5') and the *Nco*I-*Eco*RI fragment (nucleotides 3031 to 3800, designated as pNT-3') into the shuttle vector pSL301 (Invitrogen). An internal *Xba*I site found within both pNT-5' and pNT-3' facilitated further the cloning procedures. The residues Y882, Y913, Y928 and Y957 are within a 5'*Xba*I-*Nco*I3' fragment in the NT-5' fragment while Y1010 and Y1132 are within an 5'*Nco*I-*Xba*I3' fragment of the NT-3'fragment. The oligonucleotides generated to create the tyrosine-to-phenylalanine mutations are outlined in Table 2.1. Nucleotide substitutions that have been modified from the wild type have been underlined. PCR based mutagenesis to generate all mutants follow the protocol described previously (Siegel et al., 1994). All amplified products were subcloned into their appropriate target plasmid and sequenced to confirm the presence of the appropriate mutation. Standard subcloning techniques were utilized in this procedure. All nucleotide primer generation and DNA sequencing was performed by the MOBIX Central Facility of McMaster University by D. Gooden and B. Allore respectively.

2.1.3a *Generation of the TK2 chimeric*

The EGFR and TK cDNA's were released by a *Xho*I digest and placed into the shuttle vector pGEM7 (Invitrogen). Both cDNAs were digested with *SalI* and *Spe*I, and the insert released from the EGFR was ligated into pGEM7-TK to yield TK2. The resulting cDNA was then introduced into the expression vector pJ4Ω. All restriction sites engineered in the wild-type EGFR and TK cDNA constructs have been described previously (Di Fiore et al., 1987a; Di Fiore et al., 1987b; Di Fiore et al., 1990).

2.1.3b *Generation of the EGFR^{YHAD} construct*

The EGFR cDNA was released by a *Xho*I digest and introduced into pGEM7. A *Spe*I-*Acc*I digest released an insert that was then used to amplify the appropriate mutation

Table 2.1 Oligonucleotides

NT site-directed mutagenesis

YHAD-Y882F	AB9602	Forward	5'	ACA GAG TTC CAT GCA GAT GGG GGC A	3'
	AB9603	Reverse	5'	TGC ATG GAA CTC TGT CTC ATC AAT	3'
YGVT-Y913F	AB9855	Forward	5'	TGG AGC TTT GGA GTG ACT GTG TGG	3'
	AB9856	Reverse	5'	CAC TCC AAA GCT CCA CAC ATC ACT	3'
YDGI-Y928F	AB7890	Forward	5'	GCC AAA CCT TTC GAT GGA ATC CCA GCC	3'
	AB7891	Reverse	5'	TCC ATC GAA AGG TTT GGC CCC AAA AGT	3'
YMIM-Y957F	AB9857	Forward	5'	GAT GTC TTC ATG ATT ATG GTC AAA	3'
	AB9858	Reverse	5'	AAA CAT GAA GAC ATC AAT GGT GCA	3'
YRSL-Y1010F	AB9859	Forward	5'	TTC TTC CGT TCA CTG CTG GAA	3'
	AB9860	Reverse	5'	TGA ACG GAA GAA GGT ACT GTC	3'
YSED-Y1132F	AB7894	Forward	5'	CAG CGG TTC AGC GAG GAC CCC ACA	3'
	AB7895	Reverse	5'	CTC GCT GAA CCG CTG TAG AGG GCT	3'

NT site-directed mutagenesis and sequencing

XbaI-Forward	AB7888	Forward	5'	TCT CCT AAA GCC AAC	3'
NcoI-Forward	AB7892	Forward	5'	GTG GTC ATC CAG AAC GAG	3'
NcoI-Reverse	AB7889	Reverse	5'	CAG TGA ACG GTA GAA GGT	3'
EcoRI-Reverse	AB7893	Reverse	5'	CTG GTA CCA TCG ATG	3'

EGFR^{YHAD} construct

EGFR ^{YHAD}	AB13975	Forward	5'	ACA GAG TAC CAT GCA GAT GGA GGC	3'
	AB13976	Reverse	5'	TGC ATG GTA CTC TGT CTC TTC CGC	3'
SpeI-Forward	AB14599	Forward	5'	ATG AAC TAC TTG GAG GAC CGT CGA CTA GTG CAC	3'
AccI-Reverse	AB14600	Reverse	5'	GCA CTT GAC CAT GAT CAT GTA GAC ATC GAT	3'

Lightcycler primers

mEstrogen receptor α	AB25455	Forward	5'	ACA CGT TTC TGT CCA GCA CC	3'
	ABxxxx	Reverse	5'	GCC TTT GTT ACT CAT GTG CC	3'
mCyclinD1	AB25424	Forward	5'	CCC GCT GGC CAT GAA CTA C	3'
	AB25366	Reverse	5'	GTG TGT GCA TGC TTG CGG	3'
mFAK	AB25367	Forward	5'	CCT GGG CCA GTA TAA TCA	3'
	AB25368	Reverse	5'	GCA TGT AGT CAC TCT TCA	3'
mPGK	ABxxxx	Forward	5'	CAC AGA GGA TAA AGT CAG CC	3'
	ABxxxx	Reverse	5'	ATA GAC GCC CTC TAC AAT GC	3'

to generate the $EGFR^{YHAD}$ sequence. This product was then reintroduced into the parental pGEM7-EGFR vector and released as an *Xho*I insert into pBluscript digested with *Sal*I. Upon confirming the correct orientation, the unique flanking restriction sites 5' *Hind*III-*Kpn*I 3' released the $EGFR^{YHAD}$ cDNA to be ligated into the expression vector pJ4Ω.

2.2 *In vitro* association assays

2.2.1 Generation of GSTag Fusion Proteins

Overnight cultures of fusion proteins were grown in LB-Ampicillin at 37°C. These were then transferred to a fresh large-scale 100 mL culture at a 1:10 dilution and grown for one hour. The generation of the fusion protein was then induced with 100μM IPTG and incubated for an additional 3-5 hours. Cultures were pelleted and resuspended in 1/100 the culture volume in MTPBS (150 mM NaCl, 16 mM NaH_2PO_4, 4 mM Na_2HPO_4, 10 mg/mL aprotinin, 10 mg/ml leupeptin) and then sonicated twice at 5 seconds each. A final concentration of 1% TritonX-100 was added to the lysates, vortexed and cleared by centrifugation at 13000 rpm at 4°C. GSTag fusion proteins were affinity purified from the bacterial lysate by introducing glutathione sepharose beads (CLB4, Pharmacia). The lysate/bead mixture was rotated end-over-end at room temperature for 20 minutes. Beads were washed 5 times with MTPBS and stored in MTPBS buffer at 4°C. Fusion proteins were eluted by incubating the beads with excess glutathione (2 times the packed bead volume of 15 mM glutathione (Sigma); 50 mM TrisCl pH 8.0). Total protein was quantitated using the Bradford assay following the manufacturers instructions (Biorad).

2.2.2 Direct Binding Assays (Far Western)

Subsequent to the transfer to the PVDF membrane, the blot was incubated with 2□g/ml of GSTagSrcSH2 fusion protein diluted in 5% bovine serum albumin (BSA)/TBS for 3 hours at room temperature and washed 4 times in TBS for 10 minutes each. The membranes were incubated with anti-GSTag/5% BSA/TBS for 3 hours at room

temperature, washed 4 times in TBS for 10 minutes each, and incubated with horseradish peroxidase-conjugated anti-mouse antibodies for 45 minutes and washed 4 times in TBS for 10 minutes each. The membrane was exposed to ECL as specified by the manufacturer.

An alternate method to detect direct association utilized raioloabeled GSTag probes. Following protein transfer to PVDF, membranes were incubated in 5% block buffer (5% skim milk in 20mM HEPES pH 7.5; 5mM MgCl2; 1mM KCl; 5mM DTT; 10% sodium azide) for 1 hour at room temperature. Fusion proteins were washed 3 times in DK buffer (50mM KH2PO4; 10mM MgCl2; 5mM NaF; 4.5mM DTT). The fusion proteins were incubated in labeling buffer (50mM KH2PO4; 10mM MgCl2; 5mM NaF; 4.7mM DTT) with 500μCi 32Pg-ATP (6000Ci/mM) and 20μg of protein kinase A (Sigma P-2645) for 30 minutes at 30°C, after which an additional 20μg of protein kinase A was added. Unincorporated nucleotides were removed with 5 washes in ice cold 1x PBS containing 5mMNaF and 5mM EDTA. Proteins were euted as above and 1.5x106 cpm/mL of fusion in block buffer was used to probe the membrane for 3 hours at room temperature. Membranes were washed 3 times in 1x TBS-T and analyzed by autoradiography.

2.3 Cell Lines, Culture Medium and Tissue Explants

2.3.1 Cell lines, culture media and ligands

Rat1, MCF-7, NAFA (Muller et al, 1988), and R1/HER (Osherov and Levitzki, 1994) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with fetal bovine serum, penicillin, streptomycin and amphotercin B. When necessary, DMEM that does not contain phenol red was utilized (DMEM-PR, Gibco). All cell lined were maintained in 10% fetal bovine serum (FBS, Gibco) unless otherwise specified. MCF-7 ERE3 cell lines express a luciferase reporter gene under the control of a 3x estrogen response element (ERE) derived and provided by Dr. S. Dedhar.

When required to transiently stimulate cells, human recombinant epidermal growth factor (EGF, Gibco) was used at a concentration of 100 ng/mL for the required times. Cells that were maintained in the presence of EGF were cultured at a concentration of 10 ng/mL. To stimulate with estradiol (E2, Calbiochem), cells were incubated with 100nM of E2 for the required times. Serum starvation of cells consisted of incubation with DMEM supplemented with 0.5% FBS/penicillin/streptomycin/amphotercin-B.

To strip endogenous lactogens from FBS, 0.25% Norit A/0.0025% Dextran T-70 (w/v) was suspended in 10 mM Tris-HCL pH 8.0. For one mL of serum to be treated, two mL of charcoal suspension was pelleted, resuspended in serum and incubated at 55°C for 30 minutes. Subsequently, the charcoal was pelleted and the serum transferred to fresh charcoal. The process was repeated three times to remove the majority of the lactogens present in the serum. Finally, the stripped serum was sterilized using a 0.2μm pore size filter (Nalgene).

2.3.2 Preparation of tissue explants

Using sterile techniques, the inguinal mammary gland of virgin female Fvb/n mice were excised and dissected into small fragments in 1x PBS. With the addition of an equal volume of 2x collagenase/dispase solution (0.05g collagenase, Boeringer Manheim 103-586; 0.5g dispase, Boeringer Manheim 165-589;100mL of PBS, gravity filter once with Whatman and once through a 0.2μm filter), the tissue explant was incubated at 37°C for one hour under constant agitation. The solution was cleared by centrifugation at 1000 rpm and the pellet was resuspended in DMEM supplemented with 10% FBS/penicillin/streptomycin/amphotercin-B.

2.3.3 Derivation of Stable Cell Lines

Cells were cultured in DMEM supplemented with 10% fetal bovine serum with penicillin, streptomycin and amphotercin-B. All stable cell lines were established via electroporation. For each construct of interest, 10^6 cells were co-electroporated with 1 μg of cDNA with a 1kV and 25μFd electrical pulse in an electroporation cuvette (Biorad

0.4cm gap, cat# 165-2088). The final volume within the cuvette, 800μL, represented 200μL of PBS containing the plasmid(s) of interest and 600μL of the target cells resuspended in PBS. Typical time constants ranged from 0.38 to 0.40 msec. Subsequent to electroporation, cells were resuspended in sufficient media to plate 10^6cells/100mm plate. If a selectable marker was not present on the vector, a PGK-puro construct was co-electroporated at a 40:1 ratio of expression plasmid to selectable marker. Selection was initiated on Rat1 fibroblast cells with 3 μg/mL of puromycin or 0.8 μg/mL for epithelial cell types. Positive colonies were screened for expression by western blot.

2.4 Focus-forming Assays

Transformation assays were performed by electroporating a pool of cells representing 10^6 cells/plate, for every 10^6 cells, 1μg of DNA was electroporated. If necessary, a 20:1 ratio of expression plasmid to selectable marker was used. The final volume within the cuvette, 800μL, represented 200μL of PBS containing the plasmid(s) of interest and 600μL of the target cells resuspended in PBS. A 1kV and 25μFd electrical pulse was applied in an electroporation cuvette containing the cells and plasmids of interest (Biorad electroporation cuvette, 0.4cm gap, cat# 165-2088). Typical time constants ranged from 0.38 to 0.40 msec. Subsequent to electroporation, cells were resuspended in sufficient media to plate 10^6cells/100mm plate. Cells were maintained for 14 days with DMEM/10% FBS, changing the media every 3 days. The plates were washed twice with 1x PBS and fixed overnight with 10% buffered formalin. Plates were rinsed with 1x PBS and stained with Giemsa as specified by the manufacturer and then air-dried.

2.5 Matrigel assays

Clones previously picked and expanded were grown in 100mm tissue culture dishes in 10% FCS/DMEM. Matrigel (BD Biosciences) was defrosted overnight at 4°C on ice, and on the day of seeding 24 well dishes were precooled on ice and Pasteur pipettes were placed at -20°C. 330 μL of Matrigel was dispensed in each precooled well

and incubated at 37°C for one hour to solidify. Subsequently, one mL of cold 10% FCS/DMEM containing 105 cells/well were seeded and media was changed every 2 days.

2.6 RNA preparation

Flash-frozen tissue was placed in 3mL of ice-cold guanidinium thiocyanate (4M GIT, 25mM sodium citrate, 0.1M β-mercaptoethanol) until thawed and homogenized in a polytron. The homogenate was carefully layered onto 4 mL cesium chloride (5.7M, 25mM NaOAc pH 5.0) and centrifuged at 32,000 rpm at 20°C for 18 hours. The pellet was resuspended in 500μL of dH_2O treated with DEPC and precipitated at –20°C in 50μL of NaOAc and 1mL of ethanol. After centrifugation the resulting pellet was resuspended in 50μL of dH_2O and quantitated.

An alternative method utilized the reagent TRIZOL® (GibcoBRL). A brief outline of the published protocol is as follows; a small sample of tissue was placed in 1 mL of TRIZOL reagent and homogenized with a Polytron power homogenizer. Samples were incubated for 5 minutes at room temperature, after which 200 μL of chloroform was added. Tubes were shaken for 15 seconds and incubated for 5 minutes at room temperature, then centrifuged for no more than 12,000 x g for 15 minutes at 4°C. The aqueous phase was placed in a fresh tube and RNA was precipitated with 500 μL of isopropyl alcohol for 10 minutes at room temperature, then centrifuged for no more than 12,000 x g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 1 mL of 75% ethanol, left to air dry and resuspended in Rnase-free water.

2.7 Quantitative PCR/ LightCycler™

Using a fluorescencebased method to detect specific RNA products, LightCycler™ (Roche) PCR reactions was performed on prepared RNA samples as described previously. Each reaction was set up in microcapillary tubes under the following conditions: CyclinD1 7mM MgCl2; 0.5μM primers; 200ng of RNA, ERα 5 mM MgCl2; 0.3 μM primers; 400 ng RNA, PGK 6mM MgCl2; 0.5 μM primers. All reactions were in the presence of SYBR GreenI. After each elongation cycle,

fluorescence was measured during log-linear phase of amplification 1 second before the predicted melting temperature. Quantitation was performed using LightCycler v1.2 software. Conditions were optimized fort each primer pair such that one specific melting curve was obtained.

2.8 Protein analysis

2.8.1 Preparation of cell lysates

When required, cells were treated with appropriate growth factors or cell culture media prior to lysis. Adherent cells were washed twice in ice-cold 1x PBS supplemented with 1mM sodium orthovanadate. Cell lysis was mediated by either PLCγ (50mM HEPES pH 7.5, 150mM NaCl, 10% glycerol, 1% Triton-X100, 1mM EGTA, 1.5mM MgCl2, 10mM NaF, 10mM sodium pyrophosphate) or mTNE 50mM Tris pH8.0, 150mM NaCl, 1% NP-40, 10mMNaF, 10mM sodium pyrophosphate, 2mM EDTA) lysis buffers. However, co-immunoprecipitation experiments involving c-Src utilized 0.7% 3-{(cholamidopropyl)-dimethyl-ammonio}-1-propaneosulphonate (CHAPS) lysis buffer (50 mM Tris HCl pH 8.0, 0.7% CHAPS, 50mM NaCl). All lysis buffers were supplemented with 1 mM sodium orthovanadate, 10ug/ml leupeptin and 10ug/ml aprotinin. Each lysate was cleared by centrifugation for 15 minutes at 4°C. Protein quantitation was performed by Bradford assay as specified by the manufacturer (Biorad).

2.8.2 Antibodies

When required, the amount and specific antibody utilized is outlined within the figure legends. Anti-Src (7D10, Quality Biotech; v-Src Ab1, Oncogene Science), anti-EGFR (Transduction Labs), anti-phosphotyrosine (PY20, Transduction Labs); anti-EGFR (E12020, Transduction Labs); anti-Neu (Ab3, Oncogene Science); anti-GSTag (Pharmacia Biotech); anti-Estrogen receptor (Calbiochem); anti-(p)STAT3 (New England Biolabs); anti-(p)MAPK (New England Biolabs); anti-(p)GSK3β (New England Biolabs); anti-CyclinD1 (SantaCruz); anti-(p)Rb (New England Biolabs).

2.8.3 Immunoprecipitations

Immunoprecipitation analysis was performed on stable cell lines cultured on confluent plates. All plates were washed twice in ice cold PBS with 1mM sodium orthovanadate and lysed on ice. Immunoprecipitations were performed by incubating 500μg to 1.5mg of total cell lysate with 250ng to 1 μg of the appropriate antibodies and 20 μl of protein G separose equilibrated in the appropriate lysis buffer for 3 hours rotating at 4°C and subsequently washed 4 times with lysis buffer. Samples were resuspended in 2x SDS loading buffer and boiled for 5 minutes. Proteins were fractionated by SDS-PAGE.

2.8.4 Immunoblotting

Proteins were electrophoresed on SDS-polyacrylamide gels and transferred to polyvinylidene diflouride (PVDF) membranes (Immobilon-P, Millipore). Membranes were incubated in block buffer (Tris-buffered-saline (TBS)/3% skim milk) overnight at 4°C, or at room temperature for 3 hours. The membranes were probed with the appropriate primary antibody diluted 1:1000 in block buffer for 3 hours at room temperature (and washed 4 times in TBS for 10 minutes each. The membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies diluted in 3% block buffer (1:5000, Jackson Laboratories) and subsequently washed 4 times for 10 minutes in TBS and visualized by enhanced chemiluminescence (ECL, Amersham) as specified by the manufacturer.

2.9 Kinase Assays

2.9.1 Autophosphorylation assay

Subsequent to immunoprecipitating the receptor the sample was washed 3 times with lysis buffer, once with PBS, twice with 0.5M LiCl, and once with kinase buffer (20mM Tris pH7.0, 10mM $MnCl_2$). With the removal of all traces of the buffer, 10μCi of $^{32}P\gamma$-ATP in 20 ml of kinase buffer was added to the immunoprecipitate and incubated

for 5 minutes at room temperature. An equal volume of 2x SDS loading buffer was added to stop the reaction and the samples were boiled for 5 minutes and separated by SDS-PAGE.

Prior to exposure on film, gels were treated with 30% methanol/7% acetic acid for 15 minutes, 1M KOH at 45°C for 30 minutes and then in 30% methanol/7% acetic acid for 15 minutes. Gels were subsequently dried for 30 minutes at 80°C (Biorad Gel Drier) and exposed to film.

2.10 Mouse analysis

2.10.1 Wholemount analysis

The inguinal gland with some of its surrounding peripheral connective tissue was removed and stretched on a glass slide to air-dry overnight. Glands were fixed and defatted overnight in acetone and then stained in Harris hematoxylin. After staining overnight, the glands were then destained with several rounds of acid alcohol (2% HCL/70% ethanol) until the ductal architecture can be clearly seen in contrast to the now light background of the fat pad. The sample is treated briefly with 0.02% ammonium hydroxide and then transferred to 70% ethanol for several hours followed by 100% ethanol for several hours. The gland is then transferred to xylene overnight and mounted with Permount with a coverslip.

2.10.2 Tissue preparation for histology

Tissue is washed with 1x PBS to remove any traces of blood prior to fixing in cold Bouins solution (Sigma). After fixing overnight the tissues were transferred to 70% ethanol prior to processing.

2.10.3 Mammary gland transplants

Donor mice were injected intraperitonelly with 0.5 mL of 0.002% (w/v) Trypan blue (Sigma) 24 hours prior to surgery. All recipient mice were 3 weeks of age.

Recipients were anesthetized with 2.5% avertin at 0.02cc/g body weight. All transplants involved the 4th inguinal mammary gland and were first cleared by cauterizing the nipple, then the major arteries within the region. The mammary gland was removed by sharp dissection from the nipple to the lymph node. A small tissue section from the donors 4th inguinal gland was removed and placed in a pocket prepared in the cleared gland of the recipient. Whole mount analysis was performed at the appropriate timepoints as described previously.

CHAPTER 3

Mapping of the c-Src Binding Site to the Neu/c-ErbB2 Receptor Tyrosine Kinase

3.1 Introduction

Many human malignancies are caused by the deregulation of growth factor receptors in conjunction with the cytoplasmic signaling molecules that associate with them. For example, squamous cell carcinomas and adenocarcinomas have been found to express high levels of the epidermal growth factor receptor (EGFR) and the c-ErbB2 (Neu/HER2) receptor respectively (Lovekin et al., 1991; Merlino et al., 1984; Paterson et al., 1991; Slamon et al., 1989; Xu et al., 1984). One focus in basic and clinical research has been on the role of c-ErbB2 in breast and ovarian cancers. Indeed, studies have identified that approximately 30% of all breast cancer cases display an overexpression of this receptor that correlates with a poor clinical prognosis in both node positive and node negative women (Gullick et al., 1991; Lovekin et al., 1991; Paterson et al., 1991; Slamon et al., 1987; Slamon et al., 1989; Winstanley et al., 1991). Moreover, between 40-60% of Ductal Carcinomas In Situ (DICS) express high levels of c-ErbB2, however the correlation between this and the propensity for DCIS developing into overt invasive tumors is unclear.

Direct evidence for the c-ErbB2/Neu RTK in transformation stems from numerous studies in cell culture as well as mouse models. Indeed, c-ErbB2 is a potent transforming agent in NIH 3T3 cells and its overexpression has been confirmed in a number of human epithelial cell lines. Furthermore, the overexpression of c-ErbB2/Neu in the mouse mammary gland driven by the murine mammary tumor virus (MMTV) promoter induces multifocal adenocarcinomas with rapid onset suggesting strongly that the ErbB2/Neu RTK plays a direct role in the formation of mammary tumors. Upon further analysis it was discovered that the Neu RTK mediates its transforming ability through four independent yet redundant signaling pathways. For example, all four sites

signal via Ras, with the adapter molecules Grb2 and Shc associating directly with tyrosines 1144 and 1227 respectively (Dankort et al., 1997).

In addition to the Ras pathway, the c-Src protein tyrosine kinase (PTK) has been implicated to be an important mediator of receptor signaling in a variety of systems. As discussed previously, the use of dominant negative versions of c-Src or mutation of the c-Src binding sites on the PDGFRβ (Mori et al., 1993) or the CSF-1R (Alonso et al., 1995) dramatically compromises receptor function. Along with the above receptors, a number of studies have demonstrated that c-Src can play an important role in EGFR family mediated growth and transformation. For example, the overexpression of c-Src has been found to augment mitogenesis and induce cellular hyperresponsiveness in the presence of EGF (Luttrell et al., 1988; Wilson et al., 1989). Furthermore, in established tumor cell lines EGF stimulation generates a rapid and sustained increase in Src family kinase activity (Osherov and Levitzki, 1994). Consistent with the above data, c-Src has been found to act synergistically with the EGFR to induce transformation (Bouton et al., 1991; Maa et al., 1995; Tice et al., 1999). Moreover, profiles of tyrosine hyperphosphorylation on the EGF receptor have identified a number of sites that correlate with c-Src overexpression (Maa et al., 1995; Sato et al., 1995b) suggesting synergy between Src and the EGF receptor in mediating a biological response (Biscardi et al., 1999; Tice et al., 1999). Taken together, strong evidence exists for the role of Src in EGF mediated receptor signaling.

In primary human breast cancer samples at least 70% of the kinase activity in the cytosolic fraction is due to c-Src (Ottenhoff-Kalff et al., 1992). Moreover, focal tumors derived from MMTV/Neu transgenic mice display a significant increase in c-Src kinase activity (Muthuswamy and Muller, 1994), suggesting that c-Src plays an important role in Neu induced mammary epithelial cell transformation. Interestingly, this increase appears to be due to a direct and specific interaction between c-Src and tyrosine phosphorylated Neu but not with the EGFR (Muthuswamy and Muller, 1995; Osherov and Levitzki, 1994). This difference in substrate specificity found in the EGFR family may play a role in the development of tumor-specific phenotypes. Indeed, the overexpression of the

EGFR correlates with the presence of squamous cell carcinomas and glioblastomas (Merlino et al., 1984; Xu et al., 1984) while the overexpression of the ErbB2/Neu receptor correlates with the formation of adenocarcinomas (Lovekin et al., 1991; Paterson et al., 1991; Slamon et al., 1989). Furthermore, in transformation assays c-ErbB2 is 100-fold more potent in its transforming ability when compared to the EGFR (Di Fiore et al., 1987a; Di Fiore et al., 1987b). Although the evidence suggest the importance of c-Src in EGFR family mediated mitogenesis and transformation, the specific molecular mechanism of substrate specificity is unclear.

In order to dissect further the specificity displayed between c-Src and the EGFR family, and more importantly the significance of c-Src in c-ErbB2/Neu induced transformation, a site-directed mutagenesis approach in conjunction with a chimeric receptor approach was utilized. The results that follow demonstrate that the associations typical of this receptor family ie: within the carboxyl region of the receptor, do not apply to the association between c-Src and c-ErbB2/Neu. Furthermore, depending on whether c-Src associates with the receptor, differences appear to exist in the ability to activate downstream targets. Finally, evidence suggests that c-Src may play an important role in c-ErbB2 mediated transformation.

3.2 Results

3.2.1 The carboxyl terminal region does not mediate the association of Src to the Neu receptor tyrosine kinase.

It is believed that the majority of the signal output observed upon receptor activation is from the association of cytoplasmic signaling proteins to the five known autophosphorylation sites found within the carboxyl terminal region of the EGFR family (Hazan et al., 1990). Indeed, the adaptor proteins Grb2 and Shc have been mapped to Y1144 and Y1226/7 on the Neu RTK leading to the activation of the Ras pathway (Figure 3.1) (Dankort et al., 1997). In order to address systematically whether these autophosphorylation sites mediate the association of c-Src to the Neu RTK, a series of

Figure 3.1 Homology of c-ErbB2 and the EGFR.

Shown is the alignment of c-ErbB2 and the EGFR. The major tyrosine autophosphorylation sites have been indicated and labeled as A(Y1028), B(Y1144), C(Y1201), D(Y1226/1227) and E(Y1253) (Dankort et al., 1997). Non-autophosphorylation sites are indicated as black circles (Y1010 and Y1132). Tyrosine residues found within the carboxyl terminal region of the kinase domain are indicated as black triangles (Y882, Y913, Y928 and Y937).

```
c-ErbB2  683  RKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKETELRKVKVLGSGAFGTVYKGIWIPD 742
              RK T+RRLLQE ELVEPLTPSG  PNQA +RILKETE +K+KVLGSGAFGTVYKG+WIP+
   EGFR  675  RKRTLRRLLQERELVEPLTPSGEAPNQALLRILKETEFKKIKVLGSGAFGTVYKGLWIPE 734

         743  GENVKIPVAIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLLGICLTSTVQLVTQLMP 802
              GE VKIPVAIK LRE TSPKANKEILDEAYVMA V +P+V RLLGICLTSTVQL+TQLMP
         735  GEKVKIPVAIKELREATSPKANKEILDEAYVMASVDNPHVCRLLGICLTSTVQLITQLMP 794

         803  YGCLLDHVRENRGRLGSQDLLNWCMQIAKGMSYLEDVRLVHRDLAARNVLVKSPNHVKIT 862
              +GCLLD+VRE++  +GSQ LLNWC+QIAKGM+YLED RLVHRDLAARNVLVK+P HVKIT
         795  FGCLLDYVREHKDNIGSQYLLNWCVQIAKGMNYLEDRRLVHRDLAARNVLVKTPQHVKIT 854
                           ▼                                ▼
         863  DFGLARLLDIDETEYHADGGKVPIKWMALESILRRRFTHQSDVWSYGVTVWELMTFGAKP 922
              DFGLA+LL  +E EYHA+GGKVPIKWMALESIL R +THQSDVWSYGVTVWELMTFG+KP
         855  DFGLAKLLGAEEKEYHAEGGKVPIKWMALESILHRIYTHQSDVWSYGVTVWELMTFGSKP 914
              ▼                           ▼
         923  YDGIPAREIPDLLEKGERLPQPPICTIDVYMIMVKCWMIDSECRPRFRELVSEFSRMARD 982
              YDGIPA EI  +LEKGERLPQPPICTIDVYMIMVKCWMID++ RP+FREL+ EFS+MARD
         915  YDGIPASEISSILEKGERLPQPPICTIDVYMIMVKCWMIDADSRPKFRELIIEFSKMARD 974
                                     ●                          A
         983  PQRFVVIQ-NEDLGPASPLDSTFYRSLLEDDDMGDLVDAEEYLVPQQGFFCPDPAPGAGG 1041
              PQR++VIQ +E +   SP DS FYR+L++++DM D+VDA+EYL+PQQGFF
         975  PQRYLVIQGDERMHLPSPTDSNFYRALMDEEDMDDVVDADEYLIPQQGFF---------- 1024

        1042  MVXXXXXXXXXXXXXXXXXXXXEPSEEEAPRSPLAPSEGAGSDVFDGDLGMGAAKGLQSL 1101
                                     S     R+PL  S  A S+  +  +      GLQS
        1025  ----------------------SSPSTSRTPLLSSLSATSN--NSTVACIDRNGLQSC 1058
                          ●                                B
        1102  PTHDPSPLQRYSEDPTVPLPSETDGYVAPLTCSPQPEYVNQPDVRPQPPSPREGPLPAAR 1161
              P  + S LQRYS DPT  L  ++       T  P PEY+NQ  V  +P    + P+   +
        1059  PIKEDSFLQRYSSDPTGALTEDS----IDDTFLPVPEYINQ-SVPKRPAGSVQNPVYHNQ 1113
                                                 C
        1162  PAGATLERAKTLSPGKNGVVKDVFAFGGAVENPEYL--------TXXXXXXXXXXXXXXX 1213
              P           +P ++   +D      AV NPEYL
        1114  PLNP--------APSRDPHYQD--PHSTAVGNPEYLNTVQPTCVNSTFDSPAHWAQKGSH 1163
                       D                          E
        1214  XXXXDNLYYWDQDPPERGAPPSTFKGTPTAENPEYL 1249
                  DN  Y     P+   P   FKG+ TAEN EYL
        1164  QISLDNPDYQQDFFPKEAKPNGIFKGS-TAENAEYL 1198
```

tyrosine-to-phenylalanine mutants were derived in the context of the point activated Neu receptor (V664E, NeuNT) (Bargmann et al., 1986; Bargmann and Weinberg, 1988b). Each of the five tyrosines were designated from A to E in the following manner; NeuNT-A (tyrosine residue 1028 to phenylalanine), NeuNT-B (Y1144F), NeuNT-C (Y1201F), NeuNT-D (Y1226/7F) and NeuNT-E (Y1253F). In addition, a construct was generated where all five tyrosines were removed and named NYPD (NeuNT Tyrosine Phosphorylation Deficient). Furthermore, in the context of NYPD each of the five tyrosines were systematically replaced and referred to as NeuNT-YA to YE (Figure 3.2a) (Dankort et al., 1997).

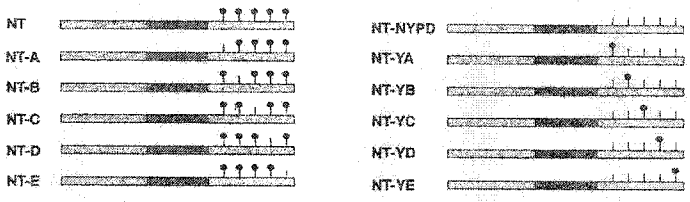
In order to address the ability of the c-Src PTK to associate with the five-autophosphorylation sites found within the carboxyl terminal region of the receptor, a direct binding assay was performed on the various NeuNT mutants. In this assay, the ability of a SH2 domain to associate with phosphotyrosine residues found on a denatured, activated receptor immobilized on a membrane was assessed. This provides a direct method to address the association between a specific SH2 domain to a tyrosine residue. The results reveal that the individual removal of any of the five autophosphorylation sites from the NeuNT receptor results in the association of the SrcSH2 fusion to the receptor, similar to the association observed with the unaltered NeuNT cDNA (Figure 3.2b, lane 1-7). The association of the SrcSH2 domain to the NeuNT-A to E mutants correlated with receptor tyrosine phosphorylation (Figure 3.2c, lane 3-7). These results suggest that the alteration of any single tyrosine residue found in the carboxyl terminal region results in the association of c-Src to the receptor and that potentially other tyrosine residues may mediate the association.

In order to identify whether any of the five tyrosines can independently mediate the association of c-Src to the NeuNT receptor, each of the NeuNT-YA to YE mutants (add-back mutants) were subjected to the same analysis preformed previously. To this end, it was observed that the SrcSH2 region still associates with each of the NeuNT YA to YE mutants at comparable levels to the unaltered NeuNT cDNA (Figure 3.2b, lane 1, 2, 9-13). Again, the association correlated with the tyrosine phosphorylation of the

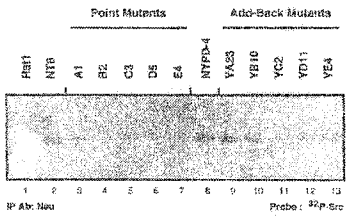
Figure 3.2 c-Src does not associate with the five-autophosphorylation sites on NeuNT.

(A) Schematic representation of the NeuNT receptor (NT) that harbors the various mutants represented by point-mutations that remove each autophosphorylation site and replaces it with phenylalanine (NT-A to E), NT with all the autophosphorylation sites removed (NT-NYPD) and the add-back mutants where each tyrosine is replaced in the context of NYPD (NT-YA to YE). (B) Each NT mutant was immunoprecipitated and the ability of a GSTagSrcSH2 fusion protein to associate directly to each receptor was assessed. (C) Neu was immunoprecipitated and subjected to an anti-phosphotyrosine immunoblot. (D) The ability of c-Src to interact with NeuNT (NT6), NYPD or a mammary cell line that expresses high levels of NeuNT were analyzed via in vivo co-immunoprecipitation.

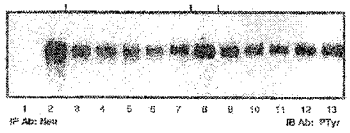
A.


NT
NT-A
NT-B
NT-C
NT-D
NT-E
NT-NYPD
NT-YA
NT-YB
NT-YC
NT-YD
NT-YE

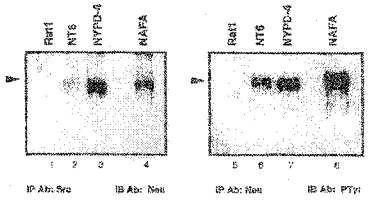
B.


Point Mutants
Add-Back Mutants
Rat1
NT6
A1
B2
C3
D5
E4
NYPD-4
YA33
YB16
YC2
YD11
YE4
IP Ab: Neu
Probe : 32P-Src

C.


IP Ab: Neu
IB Ab: PTyr

D.

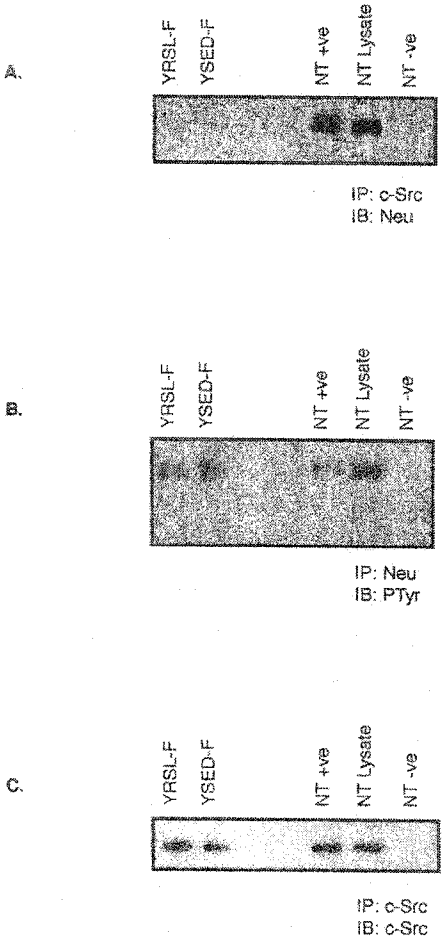

Rat1
NT6
NYPD-4
NAFA
IP Ab: Src
IB Ab: Neu
IP Ab: Neu
IB Ab: PTyr

receptor (Figure 3.2c, lane 9-13). Significantly, the analysis of the NYPD receptor revealed definitively that the autophosphorylation sites do not mediate the association of the SrcSH2 domain (Figure 3.2b, lane 8). Interestingly, the NYPD receptor retains tyrosines that are phosphorylated (Figure 3.2c, lane 8), suggesting that an interaction between the c-Src PTK and NYPD is still possible. Indeed, previous reports show that the NYPD receptor retains similar levels of catalytic activity when compared to any of the NeuNT mutants and the unaltered NeuNT receptor (Dankort et al., 1997). Consistent with the previous observation, *in vivo* co-immunoprecipitation analysis using cell lines that overexpress either NeuNT, NYPD or a mammary epithelial cell line that overexpresses NeuNT (NAFA) reveal that c-Src can clearly associate with NYPD, this association being tyrosine phosphorylation dependent (Figure 3.2d). These results further support the above observation that Src does not associate with the five tyrosines found in the carboxyl terminal region and that alternative phosphotyrosine residues mediate the association of Src to the Neu RTK.

Although the data suggests strongly that the carboxyl terminal region does not mediate the association of Src to the receptor, other non-autophosphorylation sites may play a role in providing the means of the Src PTK to associate with the Neu RTK. Indeed, two tyrosines are found within the carboxyl terminal region that may mediate such an interaction, YRSL (Y1010) and YSED (Y1132) (refer to Figure 3.1). In order to address the ability of these tyrosines to mediate the association of Src to NeuNT each site was converted to phenylalanine and subjected to an *in vivo* co-immunoprecipitation assay. The removal of these residues does not alter the ability of the Src PTK to associate with the receptor (Figure 3.3a). Furthermore, the removal of the two non-autophosphorylation sites does not alter the ability of the receptor to phosphorylate tyrosine residues by comparison to the unaltered NeuNT cDNA (Figure 3.3b). Taken together, these results suggest that the ability of the Src PTK to associate with Neu is not reliant on any of the tyrosine residues found within the carboxyl terminal region of the receptor.

Figure 3.3 c-Src does not associate with Y1010 or Y1132 on NeuNT.

Co-immunoprecipitation analysis between c-Src and NeuNT that harbor tyrosine-to-phenylalanine mutations at sites Y1010F or Y1132F. (A) c-Src was immunoprecipitated from stable cell lines that express the above mutants and immunoblotted to detect the potential association of Neu within the complex. (B) Each mutant receptor was immunoprecipitated and the content of phosphotyrosine was analyzed. (C) The conformation of equivalent levels of immunoprecipitated c-Src was assessed by immunoblotting with anti-Src.


A.
YRSL-F
YSED-F
NT +ve
NT Lysate
NT -ve
IP: c-Src
IB: Neu
B.
YRSL-F
YSED-F
NT +ve
NT Lysate
NT -ve
IP: Neu
IB: PTyr
C.
YRSL-F
YSED-F
NT +ve
NT Lysate
NT -ve
IP: c-Src
IB: c-Src

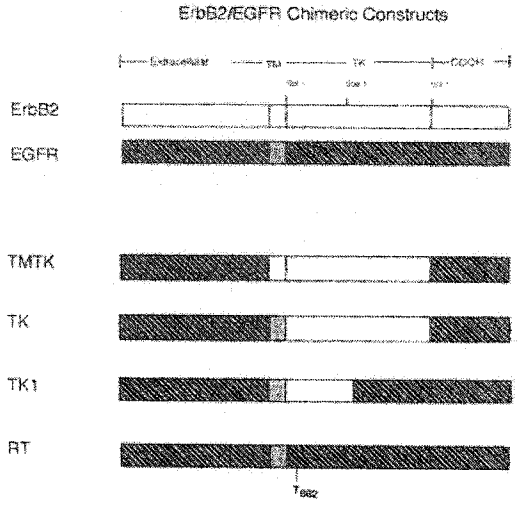
3.2.2 The carboxyl terminal region of the kinase domain in the c-ErbB2 RTK mediates the association of the Src PTK.

An issue in signal transduction is the mechanism of specificity, how do receptors provide such a diverse range of outputs given a limited number of inputs. One mechanism is for a receptor to associate with a specific subset of signaling molecules. Indeed, within the EGFR family the c-ErbB3 RTK appears to be the major partner for PI3'K association and signaling (Carraway and Cantley, 1994). Similarly, the c-Src PTK interacts specifically with Neu/c-ErbB2 and not to a close family member, the EGFR (Muthuswamy and Muller, 1995; Osherov and Levitzki, 1994). Given the differential binding observed between c-Src and the members of the EGFR family chimeric receptors of EGFR and c-ErbB2 (Figure 3.4) were derived to define further the region on Neu/c-ErbB2 necessary for c-Src association. To this end, Rat1 fibroblasts that express the chimeric receptors were derived and the association of c-Src was assessed via co-immunoprecipitation. Upon EGF stimulation, an increase in c-Src association is observed in two chimeric receptors that harbor an intact c-ErbB2 kinase domain, TMTK and TK (Figure 3.5a). The increase in association correlates with an EGF dependent increase in receptor tyrosine phosphorylation (Figure 3.5b). Furthermore, the difference in association between chimeras is not due to different levels of c-Src (Figure 3.5c). Interestingly, the chimeric receptor TK1 that is missing the carboxyl terminal region of the c-ErbB2 kinase domain does not appear to be able to associate with c-Src (Figure 3.5a, lane 7 and 8). This data suggests two possibilities; 1) the carboxyl terminal region of the c-ErbB2 kinase domain (referred to as TK2, Figure 3) physically maps the c-Src binding site or; 2) the TK2 region is necessary but does not physically map the c-Src binding site. Within this receptor class, many cytoplasmic signaling molecules dock within the carboxyl tail of the receptor. However the five known autophosphorylation sites do not mediate the association of c-Src to the Neu RTK both *in vitro* and *in vivo* (Figure 3.2). Moreover, Di Fiore *et al.* has characterized an EGFR mutant that display mitogenic and transforming properties like the c-ErbB2 receptor. This switch is mediated by a single point mutation at R662T (RT) that converts the catalytic specificity of the

Figure 3.4 Diagrammatic representation and sequence alignment of chimeric constructs.

(A) The structures of the EGFR/c-ErbB2 chimerics are shown, dark bars representing the EGFR, light bars representing the c-ErbB2 receptor. (B) Sequence alignment of the c-ErbB2 receptor and the EGFR begining from amino acid 805 on the c-ErbB2 RTK. Autophosphorylation sites are highlighted in red and are represented as follows: Y1028 (site A), Y1144 (site B), Y1201 (site C), Y1226/7 (site D) and Y1253 (site E). Non-autophosphorylation sites relevant to this study are highlighted in blue.

A.


ErbB2/EGFR Chimeric Constructs
ErbB2
EGFR
TMTK
TK
TK1
RT

B.

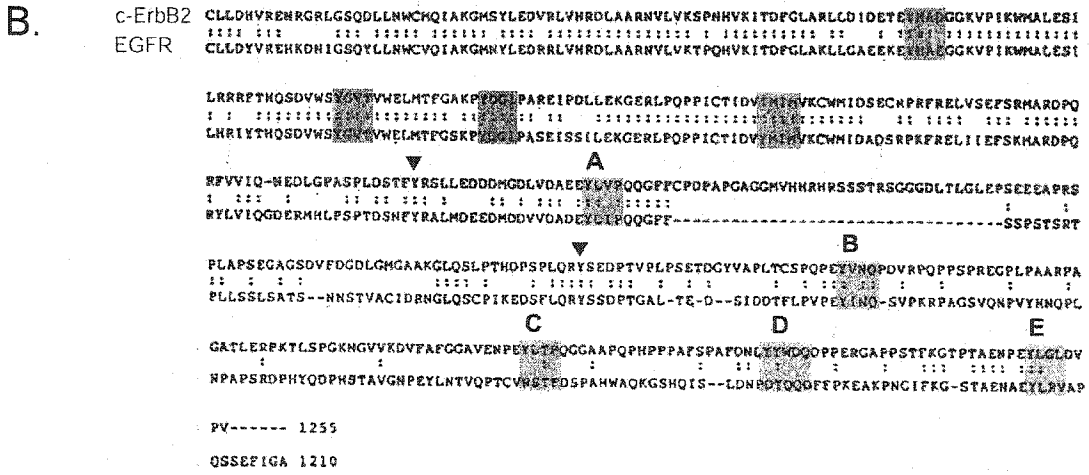
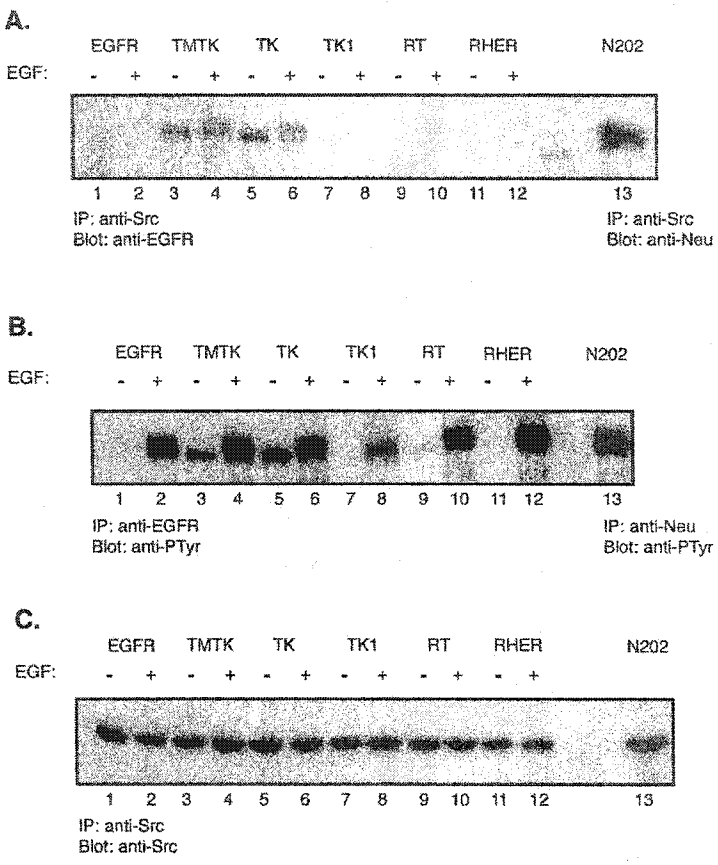

c-ErbB2
EGFR
A
B
C
D
E

Figure 3.5 c-Src associates specifically with TMTK and TK.

Co-immunoprecipitation analysis of stable cell lines expressing the various chimeric constructs. (A) c-Src was immunoprecipitated with anti-Src (7D10) from stable cell lines expressing the various chimeric constructs and the presence of the EGFR within the immunocomplex was assessed both in the absence and presence of 100ng/mL EGF for 5 minutes at room temperature. (B) The presence of tyrosine phosphorylation upon EGF stimulation on each receptor was analyzed by immunoprecipitating the chimeric receptors and immunoblotting with anti-phosphotyrosine (PY20). (C) The levels of c-Src in each immunoprecipitation was analyzed by immunoblotting with anti-Src (Ab1).


A.
EGFR TMTK TK TK1 RT RHER N202
EGF: - + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12 13
IP: anti-Src
Blot: anti-EGFR
IP: anti-Src
Blot: anti-Neu
B.
EGFR TMTK TK TK1 RT RHER N202
EGF: - + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12 13
IP: anti-EGFR
Blot: anti-PTyr
IP: anti-Neu
Blot: anti-PTyr
C.
EGFR TMTK TK TK1 RT RHER N202
EGF: - + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12 13
IP: anti-Src
Blot: anti-Src

EGFR to that resembling the c-ErbB2 receptor (Di Fiore et al., 1992). Significantly, there was no detectable association between c-Src and the RT mutant (Figure 3A, lane 9 and 10). These data suggest that the kinase region proximal to the carboxyl terminal region of the receptor mediate the association between the c-Src PTK and the c-ErbB2 receptor.

3.2.3 The TK2 region can mediate the association of c-Src to c-ErbB2

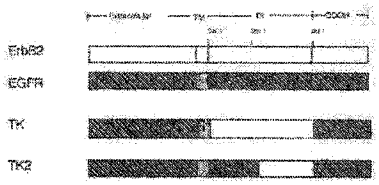
In order to further dissect the region that is necessary in mediating the association of c-Src to c-ErbB2, a complementary chimeric designated TK2 (Figure 3.6) was derived that harbors the region from the c-ErbB2 receptor that is suspected to provide the ability of c-Src to associate with the TMTK and TK chimeric constructs. Co-immunoprecipitation analysis shows that c-Src associates with the TK2 region and that this association correlates with an EGF dependent increase in receptor tyrosine phosphorylation (Figure 3.6 a and b). The difference in association between cell lines is not dependent on the levels of immunoprecipitated c-Src (Figure 3.6c). Furthermore, a direct binding assay that utilizes a c-SrcSH2 fusion protein suggests that the association mediated by the TK2 region is direct and that this association correlates with an EGF dependent increase in tyrosine phosphorylation (Figure 3.7 a and b). Since no cytoplasmic signaling molecules have been found to associate with any tyrosine residues in the juxtamembrane region of the subclass I family of RTKs as well as the fact that previous evidence identified the TK1 region as the domain that confers receptor specificity (Di Fiore et al., 1992), the above data strongly suggests that c-Src associates directly within the TK2 region of the kinase domain.

3.2.4 Association of Src to ErbB2 within TK2 is mediated by tyrosine 882.

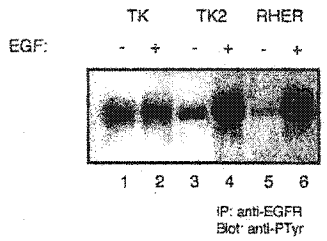
The regulation of kinase activity in receptors such as the IR and FGFR is dependent on the phosphorylation of tyrosines found within the activation loop. Phosphorylation of these tyrosines generates an open conformation leading to ATP and substrate access into the catalytic region. Interestingly, the homologous site on the EGFR differs in its +3 position which is critical for SH2 mediated specificity. Furthermore,

Figure 3.6 c-Src associates specifically with the TK2 region of the catalytic domain of the c-ErbB2 RTK.

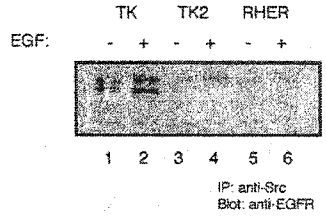
Co-immunoprecipitation analysis of stable cell lines that express the chimeric constructs were performed. (A) The ability to tyrosine phosphorylate the chimeric receptors in the absence and presence of EGF was assessed by immunoprecipitating the chimeric receptors with anti-EGFR and immunoblotting with anti-phosphotyrosine. (B) The ability of c-Src to co-immunoprecipitate with each chimeric receptor was assessed in the absence and presence of EGF. (C) The levels of c-Src that was immunoprecipitated within each experiment was assessed by immunoblotting the same blot represented in panel B with anti-Src.


Erb82
EGFR
TK
TK2

A.


TK
TK2
RHER
EGF:
- + - + - +
1 2 3 4 5 6
IP: anti-EGFR
Blot: anti-PTyr

B.


TK
TK2
RHER
EGF:
- + - + - +
1 2 3 4 5 6
IP: anti-Src
Blot: anti-EGFR

C.

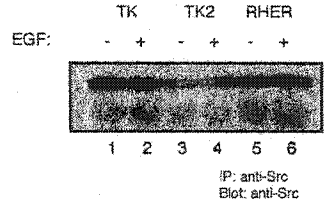
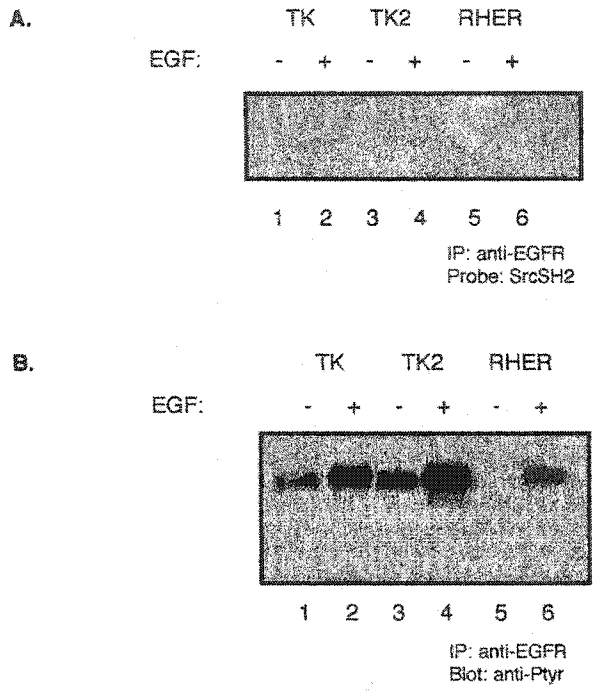

TK
TK2
RHER
EGF:
- + - + - +
1 2 3 4 5 6
IP: anti-Src
Blot: anti-Src

Figure 3.7 The c-SrcSH2 domain associates directly and specifically with the TK2 region of the c-ErbB2 RTK.

(A) The TK2 chimeric was immunoprecipitated from Rat1 stable cell lines with anti-EGFR and its ability to directly associate with a GSTagSrcSH2 fusion protein was assessed in the absence and presence of 100ng/mL EGF for 5 minutes at room temperature. (B) The ability of each receptor to become tyrosine phosphorylated was assessed via immunoblotting with anti-phosphotyrosine.


A.
TK
TK2
RHER
EGF:
- + - + - +
1 2 3 4 5 6
IP: anti-EGFR
Probe: SrcSH2
B.
TK
TK2
RHER
EGF:
- + - + - +
1 2 3 4 5 6
IP: anti-EGFR
Blot: anti-Ptyr

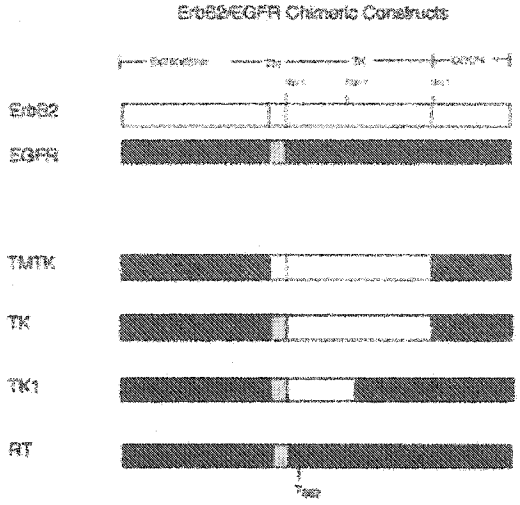
within this region all other amino acids that flank their tyrosines are homologous except for Y882. To this end, one strategy undertaken in order to circumvent this problem was to take advantage of the fact that c-Src specifically associates with c-ErbB2 and not to the EGFR. The putative binding site on c-ErbB2, Y882, including its flanking amino acids was engineered in the context of the wild type EGFR (Figure 3.8). One prediction would be that with this modified EGFR, c-Src would now associate with the EGFR when previously it could not. Significantly, upon EGF stimulation we find that while wild type EGFR does not appear to associate with c-Src, the modified receptor, termed $EGFR^{YHAD}$, now has the ability to specifically coimmunoprecipitate c-Src (Figure 3.9a). This association correlates with EGF mediated tyrosine phosphorylation of the receptor (Figure 3.9b), suggesting that the Y882 represents the binding site of c-Src to c-ErbB2/Neu.

3.2.5 Receptor phosphorylation and MAPK activation via the EGFR and $EGFR^{YHAD}$ receptor upon stimulation with EGF

While the data suggests that differences exist in the ability of specific cytoplasmic proteins to associate with wild type EGFR and $EGFR^{YHAD}$, it is unknown whether these differences translate into changes in receptor activation and downstream signaling. One pathway that has been extensively studied upon EGF stimulation is the Ras/MAPK pathway. Specifically, the ErbB2 molecule has the ability to activate the Ras/MAPK pathway utilizing multiply redundant mechanisms. Furthermore, mutation analysis on the known autophosphorylation sites resulted in no significant difference in kinase activity and the ability to phosphorylate exogenous substrates (Dankort et al., 1997). However, it is known that subtle structural differences within the kinase domain can introduce changes in substrate specificity and downstream signaling (Chang et al., 1995; Di Fiore et al., 1992; Di Fiore et al., 1990; Fazioli et al., 1991). Thus at the qualitative level, redundant transduction pathways and associated kinase activity appear similar, however differences do exist that translate into differences in biochemical and biological phenotypes.

Figure 3.8 Amino acid sequence within the TK2 region of the kinase domain.
(A) Within the TK2 region four tyrosines are found, Y882, Y913, Y928 and Y937.Within this amino acid stretch there is a high degree of homology surrounding each tyrosine residue. However we find that the sequences that flank Y882 deviates, generating within the context of the c-ErbB2 receptor an acidic region. (B) To take advantage of the differential association of c-Src for the EGFR family, a subtle receptor chimera was generated surrounding Y882 to mimic the c-ErbB2 sequence within the context of the EGFR.

A.


ErbB2/EGFR Chimeric Constructs
ErbB2
EGFR
TMTK
TK
TK1
RT

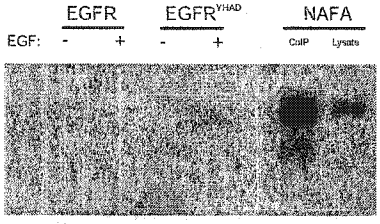
B.


c-ErbB2
EGFR
A
B
C
D
E

Figure 3.9 Flanking amino acids surrounding Y882 on c-ErbB2 can rescue the association of c-Src to the EGFR.

(A) c-Src was immunoprecipitated from stable cell lines expressing each receptor and the presence of the EGFR within the immunocomplex was assessed both in the absence and presence of EGF. (B) The presence of tyrosine phosphorylation upon EGF stimulation on each receptor was analyzed by immunoprecipitating each receptor and immunoblotting with anti-phosphotyrosine. (C) The levels of c-Src in each immunoprecipitation was analyzed by immunoblotting with anti-Src.

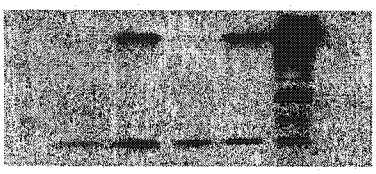
A.



IP: c-Src
IB: EGFR

IP: c-Src
IB: Neu

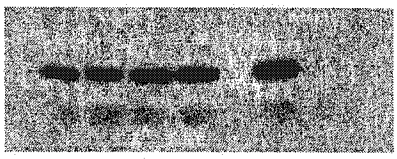
B.



IP: EGFR
IB: PTyr

IP: Neu
IB: PTyr

C.



IP: c-Src
IB: c-Src

In order to answer whether there are differences in receptor activation upon stimulation with EGF via the wild type EGFR, TK and the $EGFR^{YHAD}$, cell lines were stimulated with EGF at various timepoints and the ability to phosphorylate the receptor was assessed (Figure 3.10 a, b, and c). Within 5 seconds we observe a strong and sustained increase in receptor phosphorylation in all three receptors. This suggests that differences in receptor activation when either the c-ErbB2 kinase region as found in the TK receptor, or the mutation that defines the activation loop as found in the $EGFR^{YHAD}$ receptor, does not alter the phosphotyrosine profile of the receptor upon EGF stimulation.

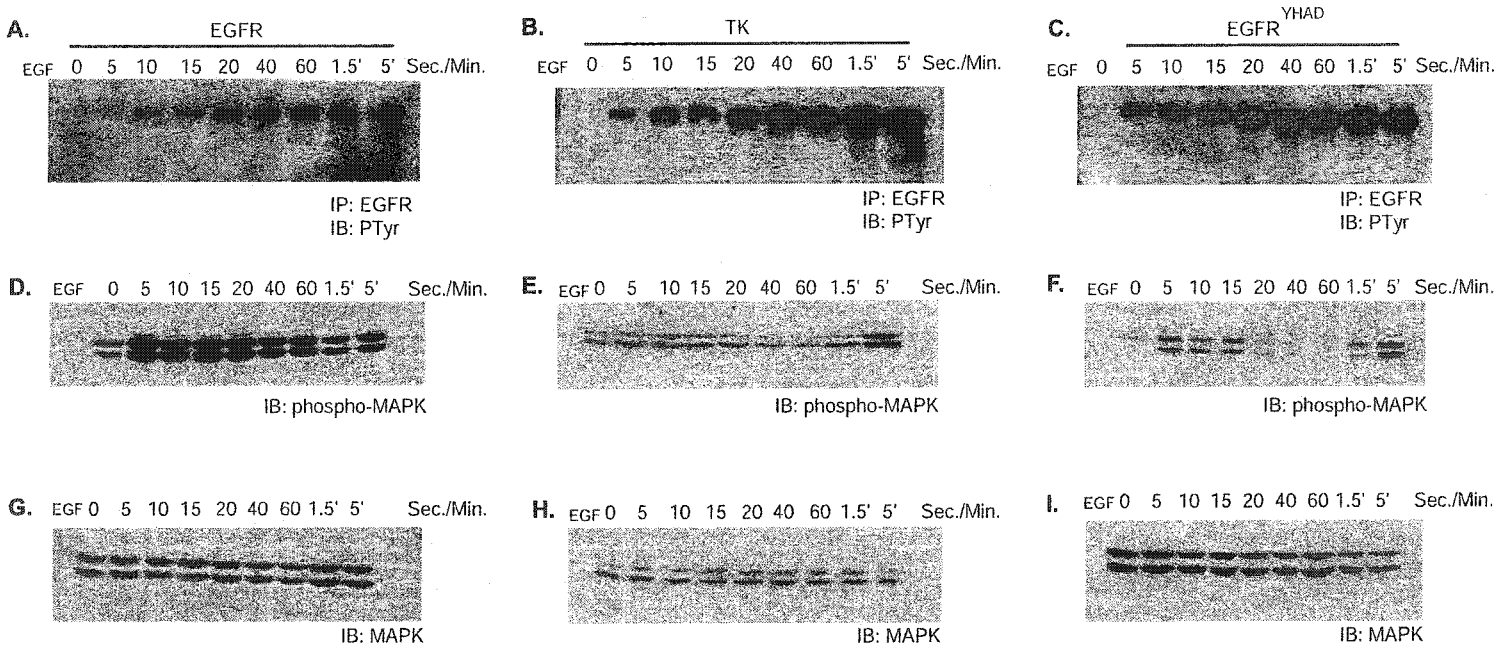
To address whether there are differences in MAPK activity upon stimulation with EGF via the wild type EGFR and the $EGFR^{YHAD}$, cell lines were stimulated with EGF at various timepoints and MAPK phosphorylation was assessed. Interestingly, stimulation of a cell line overexpressing wild type EGFR displays a rapid response to EGF simulation that declines in a temporally dependent fashion (Figure 3.10d). In contrast, stimulation of the $EGFR^{YHAD}$ cell line with EGF results in a biphasic MAPK response, characterized by a rapid response followed by a decrease and a subsequent peak at 5 minutes (Figure 3.10e). Significantly, this biphasic profile of MAPK activation is also observed in the chimeric TK receptor (Figure 3.10f). The data suggests that a very subtle mutation within the kinase region that has been found to confer the differential association between the c-ErbB2 RTK and the EGFR to c-Src may also translate into differences in downstream signaling via MAPK.

3.2.6 Differences in growth characteristics displayed by cells that express either EGFR or $EGFR^{YHAD}$ receptors

Given the differences in receptor function described above, it is possible that receptor activation may translate into distinct biological phenotypes. Indeed, the biochemical differences and biological significance between c-ErbB2 and the EGFR have been well documented with regards to transformation (Di Fiore et al., 1987a; Di Fiore et al., 1987b) and development (Britsch et al., 1998; Morris et al., 1999; Threadgill et al., 1995). Furthermore, the expression of the EGFR or the c-ErbB2 receptor within the

Figure 3.10 Differential phosphorylation of MAPK between wild type EGFR, TK, and EGFR^{YHAD} upon EGF stimulation.

Rat1 cell lines that overexpress either wild type EGFR (A, D, and G), TK (B, E, and H) or the altered receptor EGFR^{YHAD} (C, F, and I) were stimulated with 100ng/mL EGF and lysed at various timepoints. Receptors were immunoprecipitated with anti-EGFR (E12020) and immunoblotted with anti-phosphotyrosine (PY20) to assess receptor phosphorylation at each timepoint. Lysates were also subjected to anti-phosphoMAPK and anti-MAPK to assess activity.


A.
EGFR
EGF 0 5 10 15 20 40 60 1.5' 5' Sec./Min.
IP: EGFR
IB: PTyr
B.
TK
EGF 0 5 10 15 20 40 60 1.5' 5' Sec./Min.
IP: EGFR
IB: PTyr
C.
EGFR YHAD
EGF 0 5 10 15 20 40 60 1.5' 5' Sec./Min.
IP: EGFR
IB: PTyr
D.
EGF 0 5 10 15 20 40 60 1.5' 5' Sec./Min.
IB: phospho-MAPK
E.
EGF 0 5 10 15 20 40 60 1.5' 5' Sec./Min.
IB: phospho-MAPK
F.
EGF 0 5 10 15 20 40 60 1.5' 5' Sec./Min.
IB: phospho-MAPK
G.
EGF 0 5 10 15 20 40 60 1.5' 5' Sec./Min.
IB: MAPK
H.
EGF 0 5 10 15 20 40 60 1.5' 5' Sec./Min.
IB: MAPK
I.
EGF 0 5 10 15 20 40 60 1.5' 5' Sec./Min.
IB: MAPK

epithelial cell has been attributed to define acini development (Muthuswamy et al., 2001). A ligand that interacts with the EGFR family, neuregulin has been shown to play a significant role in the differentiation and growth of epithelial cells both in vitro and in vivo. The receptors that associate with neuregulin, c-ErbB3 and c-ErbB4, most likely mediate the transmission of intracellular signals that define the development of the mammary gland. However, cross-talk with c-ErbB2 as a coreceptor may increase the signaling capacity and complexity of neuregulin induced mammary gland development.

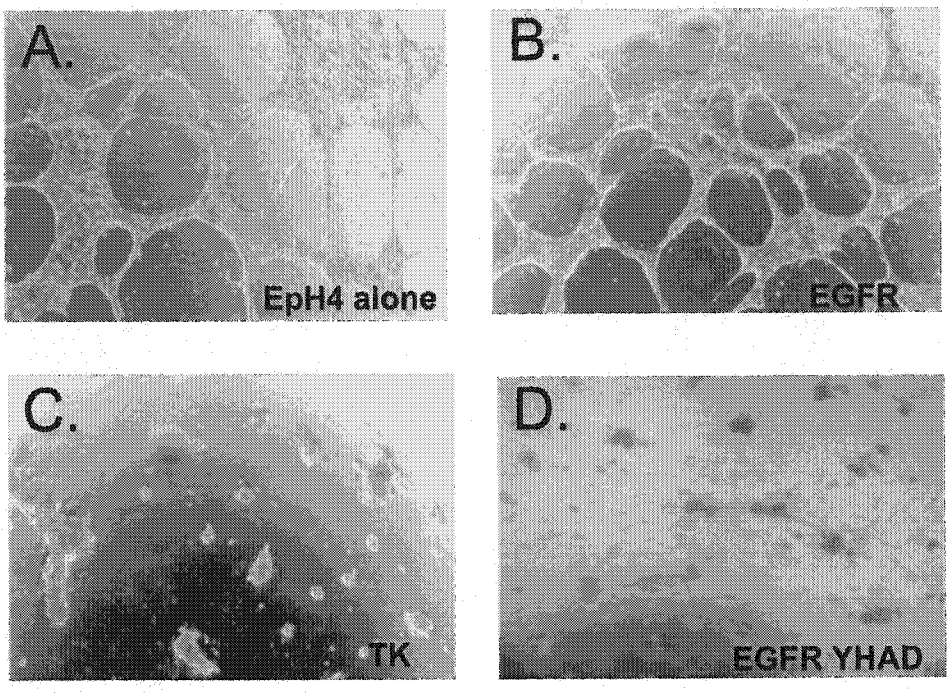
To experimentally manipulate and regulate the growth of mammary gland structures, a mammospheric technique can be utilized whereby mammary epithelial cells are cultured in matrigel under specific growth conditions resulting in the formation of complex three-dimensional structures. In the presence of growth factors such as hepatocyte growth factor/scatter factor (HGF/SF) or neuregulin, tubular or spheroid structures develop respectively, that resemble ones formed during mammary gland development. In order to assess the role of wildtype EGFR, TK and $EGFR^{YHAD}$ in mammary gland development, EpH4 cells, a mouse mammary epithelial cell line, were transfected with either EGFR, TK or $EGFR^{YHAD}$ and stable cell lines were established. Cells that were seeded in matrigel displayed growth differences as early as two days after seeding. Interestingly, while EGFR did not appear to have any significant development of mammary-like structures (Figure 3.11b), EpH4 cells that harbored either TK or $EGFR^{YHAD}$ developed a significant number of spheroidal, alveolar structures (Figure 3.11c and d). This suggests that the subtle change engineered within $EGFR^{YHAD}$ can influence the development of mammary gland structures *in vitro* similar to the catalytic region of c-ErbB2 found within the TK receptor.

3.2.7 Tyrosine 882 within the TK2 region is necessary for transformation.

Previous data regarding other receptors such as the PDGFRβ and the CSF-1R demonstrate that the ablation of the c-Src binding site compromises the catalytic activity of the receptor, suggesting that the association of c-Src to these receptors is critical for full receptor function (Alonso et al., 1995; Mori et al., 1993). Within the TK2 region of

Figure 3.11 Morphogenic differences between wild type EGFR, TK, and $EGFR^{YHAD}$ upon EGF stimulation.

EpH4 cell lines were derived that express wild-type EGFR, TK or the $EGFR^{YHAD}$ receptor. Cells were seeded onto matrigel without EGF for two days. The ability to generate spheroidal, alveolar structures was assessed.


A.
EpH4 alone
B.
EGFR
C.
TK
D.
EGFR YHAD

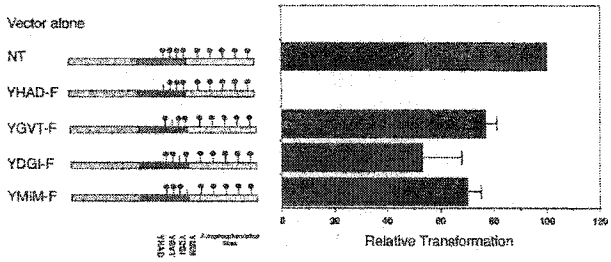
the c-ErbB2 RTK four tyrosines exist that may potentially mediate the direct association of c-Src to the RTK. In order to address the significance of these tyrosines on the Neu/c-ErbB2 RTK, site directed mutagenesis converted each tyrosine to phenylalanine in the context of NeuNT. Transformation assays were performed to assess the biological properties of each mutant receptor, (Figure 3.12). From this data it was found that the mutation of the site YHAD-F (Y882F, Y845 on the EGFR) compromises the ability of the Neu RTK to induce transformation. Conversely, YGVT-F (Y913F), YDGI-F (Y928F) and YMIM-F (Y957F) display comparable abilities to induce focus formation relative to NeuNT. Recent evidence suggests that the conversion of the YHAD site compromises the catalytic activity of the Neu RTK (Zhang et al., 1998), consistent with the data derived from the focus forming assay. In addition, previous groups have identified the YHAD site as a major non-autophosphorylation site within the TK2 region (Sato et al., 1995a; Sato et al., 1995b). Furthermore, the c-Src kinase appears to generate its own tyrosine binding site and is not dependent on the receptor itself to phosphorylate a potential site of association (Stover et al., 1995). Interestingly, the homologous site on the EGFR differs in its +3 position which is critical for SH2 mediated specificity. The above evidence in conjunction with the described data regarding the chimeric studies suggests that the YHAD site is a strong candidate for mediating the association of c-Src to Neu/c-ErbB2.

3.2.8 c-Src plays a role in Neu mediated transformation.

To assess the importance of c-Src in Neu induced transformation a dominant negative form of c-Src was used. To this end, a truncation mutant of c-Src that is missing the kinase and carboxyl terminal regulatory region designated Src251 was introduced into an expression vector (pBPΩ) containing a puromycin resistance gene and stable cell lines were generated (Figure 3.13a). The expression of Src251 does not appear to be lethal since there were no significant differences in the number of puromycin resistant colonies between the pBPΩSrc251 transfected pool versus the pBPΩ transfected pool after selection (data not shown). The ability of a clonal Src251 cell line to support focus

Figure 3.12 Tyrosine 882 within the TK2 region of the Neu RTK is critical for receptor function.

Site directed mutagenesis was performed on the four tyrosines that are found within the TK2 region, YHAD-F (Y882F), YGVT-F (Y913F), YDGI-F (Y928F) and YMIM-F (Y957F), and their ability to transform was assessed via focus formation assay.



Expression Plasmid	Focus Assay #1 Avg.# Foci [b]	Focus Assay #1 % Transform. Rel. to NT [c]	Focus Assay #2 Avg.# Foci [b]	Focus Assay #2 % Transform. Rel. to NT [c]	Relative Transforming Ability [d]
pJ4Ω	0	0	0	0	0
pJ4Ω NT	166±23	100	125±11	100	100
pJ4Ω YHAD-F	0	0	0	0	0
pJ4Ω YGVT-F	120±19	72	100±8	81	77±4
pJ4Ω YDGI-F	113±9	68	48±5	39	53±15
pJ4Ω YMIM-F	125±18	75	80±45	65	70±5

[a] Two independent focus assays were performed with established Rat-1 fibroblasts. In each case, the cDNA of interest was placed under the control of the Moloney murine leukemia virus long terminal repeat. The transforming ability of each mutant was compared with that of an activated form of Ras (NT). The expression of the empty vector was also included as a negative control for transformation.

[b] Values are the mean numbers of foci counted ± their SEMs ± standard deviations.

[c] Values are the ratios of the mean numbers of foci obtained for each construct with respect to the activated form of Ras (NT).

[d] Values are the mean transforming abilities from two experiments ± standard deviation.

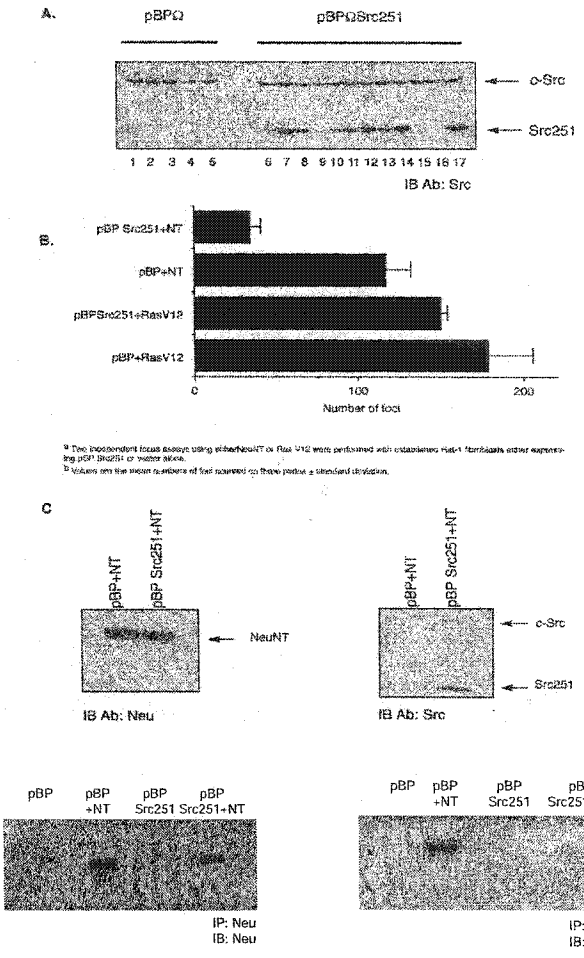
formation by NeuNT was assessed. Evidence shows that there is a significant decrease in the ability of NeuNT to transform when in the presence of Src251 (Figure 3.13b). The decrease in focus formation due to Src 251 was not due to differences in the levels of transfected NeuNT (Figure 3.13c). Significantly, in the presence of Src251 the phosphorylation of NeuNT was reduced relative to the transfected NeuNT cell line that did not express Src251 suggesting that c-Src can impinge on the efficient phosphorylation of the Neu receptor (Figure 3.13c).

Although c-Src does appear to play a role in NeuNT mediated transformation the difference in the transforming ability of NeuNT in the presence of Src251 may be due to a general inability of the Src251 cell line to support focus formation. To address this possibility RasV12 was introduced into pBPΩ alone and pBPΩSrc251 cell lines and the ability to support foci was assessed. To this end RasV12 displayed comparable levels of transformation in both the pBPΩSrc251 stable cell line and the control cell line pBPΩ alone (Figure 3.13b). In addition, it is possible that the overexpression of any SH2 containing protein may inhibit transformation mediated by NeuNT. To address this a cell line that overexpresses the adaptor protein Shc (designated ShcHA) was derived and focus-forming assays were again performed. The ability to transform was comparable between pBPΩ alone and pBPΩShcHA further suggesting that c-Src plays a role in Neu mediated transformation (data not shown).

3.3 Discussion

Approximately 30% of human breast cancers are found to have amplified and/or overexpress the c-ErbB2/Neu receptor tyrosine kinase, this overexpression having been found to inversely correlate with a positive prognosis of the patient. Under normal physiological conditions, this receptor class, like many, have the ability to associate with specific intracellular signaling molecules that upon receptor activation can elicit various cellular responses such as cell growth, differentiation or survival. However, if unchecked, these same molecules can lead to unregulated cellular growth resulting in transformation and tumorigenesis. A number of cytoplasmic molecules that promote mitogenesis have

Figure 3.13 c-Src plays an important role in NeuNT mediated transformation.
(A) Stable Rat1 cell lines that express a dominant negative version of c-Src (Src251) were generated and (B) their ability to support focus formation in the presence of NeuNT or RasV12 was assessed. (C) Plates from the focus assay were lysed and the levels of Neu as well as its level of phosphorylation both in the absence and presence of Src251 was assessed by immunoprecipitation (anti-Neu; Ab3) and immunoblotting (anti-Neu, Ab4; anti-phosphotyrosine, PY20).


A.
pBPΩ
pBPΩSrc251
c-Src
Src251
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
IB Ab: Src
B.
pBP Src251+NT
pBP+NT
pBPSrc251+RasV12
pBP+RasV12
0
100
200
Number of foci
C
pBP+NT
pBP Src251+NT
NeuNT
IB Ab: Neu
c-Src
Src251
IB Ab: Src
pBP
pBP +NT
pBP Src251
pBP Src251+NT
IP: Neu
IB: Neu
IP: Neu
IB: PTyr

been mapped to specific tyrosine residues on the c-ErbB2 receptor such as the Grb2 and Shc adaptor proteins that associate with tyrosines 1144 and 1226/7 respectively. Furthermore, the c-Src protein tyrosine kinase has been implicated to account for a high proportion of the kinase activity found in the cytosolic fraction of cells derived from samples from human primary breast cancers. Moreover, a significant increase in c-Src kinase activity is found in tumors derived from MMTV/Neu transgenic mice. Taken together, the c-Src PTK appears to play a role in the development of mammary gland transformation however the specific mechanism is unclear.

In order to further define the role of c-Src in c-ErbB2/Neu mediated transformation the physical mapping of the site of association of c-Src to c-ErbB2/Neu was undertaken. To accomplish this, site directed mutagenesis was performed on the Neu RTK and the ability of c-Src to associate to the mutants was assessed. To this end, it was found that c-Src does not associate with any of the phosphotyrosine residues found within the tail of the Neu RTK. In order to narrow down the region that mediates the association between c-Src and the c-ErbB2/Neu RTK a chimeric receptor strategy was initiated. The rationale for this strategy being that the c-Src PTK has been found to display a direct and specific association with c-ErbB2/Neu and not to the EGFR. Using different chimeras it was discovered that the region that mediates the association of c-Src to the c-ErbB2/Neu RTK was the carboxyl terminal region of the kinase domain of the c-ErbB2 receptor defined as TK2. Upon further analysis of the TK2 region four potential tyrosines were identified to possibly mediate the association of c-Src to the c-ErbB2/Neu RTK. Previous data regarding other receptors such as the PDGFRβ and the CSF-1R show that the ablation of the c-Src binding site compromises the catalytic activity of the receptor suggesting that the association of c-Src to the receptor is critical for full receptor function. In order to address whether any of the four tyrosines found within the TK2 region displays the same characteristic each tyrosine was converted to phenylalanine and their ability to transform was investigated. Interestingly, one of the tyrosines was found to be critical for transformation termed YHAD (Y882). Furthermore, the biological significance of the c-Src PTK in Neu mediated transformation became apparent with the

use of a dominant negative version of c-Src called Src251. Transformation assays reveal that in the presence of the Src251 molecule the ability of NeuNT to transform is significantly compromised. Taken together the c-Src PTK has the ability to associate within the TK2 region directly and specifically. Furthermore the ablation of tyrosine 882 within the TK2 region correlates with the ability of c-Src to associate within that region. Moreover, c-Src has been found to play a critical role in Neu mediated transformation.

3.3.1 *Physical mapping of c-Src to TK2*

Although the data suggest that the TK2 region mediates directly the association of c-Src to the c-ErbB2/Neu RTK, one can argue that the TK2 region may act to mediate the association of c-Src to another region i.e. c-Src does not physically map to the TK2 region. However, previous data suggest that the TK1 region can confer receptor specificity within the EGFR family. In addition, the specificity of the EGFR has been found to be influenced by a point mutation within the EGFR at amino acid 662 that converts an arginine to a threonine (Di Fiore et al., 1992). This mutant referred to as RT (Figure 3) has been previously characterized to display properties that are more c-ErbB2-like rather than its parental EGFR as defined by its mitogenicity, transformation ability and the profile of tyrosine phosphorylated substrates. Indeed, other kinase domains have been found to display substrate specificity changes and transforming potential as a result from modifications of their catalytic specificity (Chang et al., 1995; Pandit et al., 1996). We have shown by co-immunoprecipitation analysis that the c-Src molecule does not associate with the RT mutant. If the specificity of the kinase region of the $EGFR^{R662T}$ did shift to selectively phosphorylate tyrosines associated with c-ErbB2/Neu receptor then one possible outcome would be that a tyrosine on the RT mutant might now mediate the association between itself and c-Src. However this appears to not be the case, further suggesting that the association between c-Src and the TK2 region is direct. This also implies that the tyrosine that mediates the interaction with c-Src does not exist on the EGFR however it is present on the c-ErbB2/Neu RTK. Interestingly, upon closer inspection of the amino acid homology that flank each tyrosine shared between the c-

ErbB2/Neu RTK and the EGFR within the TK2 region, only Y882 differs in its +3 position. Significantly, this position has been previously identified as a critical factor in mediating the specificity of the SH2 module to associate with specific phosphotyrosines (Eck et al., 1993; Waksman et al., 1993). This observation is consistent with the RT co-immunoprecipitation data suggesting further that the c-Src PTK associates directly within the TK2 region of the c-ErbB2/Neu receptor.

3.3.2 *c-Src and the architecture of the kinase domain*

The data suggests that the kinase region of the c-ErbB2 RTK is involved in mediating the association of c-Src. Does the general architecture of the catalytic region facilitate the association of c-Src to the c-ErbB2/Neu RTK leading to its activation? The kinase regions of the EGF receptor family can be divided into two distinct domains based on alignment (Segatto et al., 1991). The TK1 region defined by amino acids 657 to 808 on the EGFR shares relatively less homology between all EGFR family members relative to the TK2 region defined by amino acids 809 to 927 on the EGFR. The ATP binding pocket and the activation loop are found in the TK1 and TK2 region respectively (Segatto et al., 1991). A molecular model based on the FGFR and IR for full catalytic activation involves the tyrosine phosphorylation of the activation loop which acts much like a molecular gate that permits ATP to enter the binding pocket (Weiss and Schlessinger, 1998). Indeed, the activation loop of the insulin receptor harbors three tyrosine residues that are important for the stabilization of the activation loop leading to ATP binding, substrate phosphorylation and full receptor activation (Hubbard et al., 1998). Significantly, Y882 on the Neu/c-ErbB2 RTK represents the tyrosine found on the activation loop. Moreover, if Y882 is mutated to phenylalanine the catalytic activity of the receptor is severely compromised (Qian et al., 1999). Previous observations suggest that the overexpression of c-Src results in the hyperphosphorylation of tyrosine residues that includes the activation loop (Biscardi et al., 1999; Sato et al., 1995a). In conjunction with our data that shows a direct association of c-Src to a region of the c-ErbB2/Neu RTK that includes the activation loop suggests that c-Src is one of the substrates that may

induce the full catalytic activity of the c-ErbB2/Neu receptor via activation loop phosphorylation. One implication of this model is that the c-Src PTK is upstream of the c-ErbB2/Neu RTK. Interestingly, *in vitro* data suggests that c-Src has the ability to generate its own binding site on the c-ErbB2 RTK upon which it would then associate with the receptor (Stover et al., 1995; Wasilenko et al., 1991).

In addition to the known autophosphorylation sites found in the carboxyl terminal region of the receptor, a number of non-autophosphorylation sites have also been identified within the TK2 region (Sato et al., 1995a; Sato et al., 1995b; Stover et al., 1995; Tice et al., 1999; Wasilenko et al., 1991; Wright et al., 1996). Of these, YDGI (Y928 on the Neu RTK) has been identified previously to mediate the interaction of c-Src to the EGFR (Stover et al., 1995). However, our data suggests that on the Neu RTK this tyrosine does not mediate the interaction with c-Src *in vivo* (data not shown). Moreover, Sato *et al.* failed to identify the phosphorylation of YDGI *in vitro* (Sato et al., 1995a). Interestingly, YHAD is homologous to Y416 on the v-Src kinase region, a known autophosphorylation site that modulates the catalytic activity of c-Src (Superti-Furga, 1995). Studies that have investigated the substrate specificity of various tyrosine kinases have suggested that individual kinases that contain SH2 domains prefer to phosphorylate tyrosine residues that it itself can associate with (Zhou S, 1995). Given the fact that Y882 on the Neu receptor displays a high degree of homology to Y416 found on the c-Src kinase domain, in addition to data that suggest that c-Src can generate its own site of association on the receptor (Stover et al., 1995; Wasilenko et al., 1991), suggests that the c-Src PTK may select this tyrosine to phosphorylate and thus mediate its association to the receptor. Again, the data described above is consistent with our data that suggests that c-Src can directly and specifically interact with the TK2 region of c-ErbB2/Neu where the activation loop is located. The association of c-Src to the catalytic region of a receptor is not novel. Indeed, a similar association has been previously described within the PDGFβ-receptor system, in that c-Src can phosphorylate and associate with Y934 (Hansen et al., 1996). Mutation of Y934 to phenylalanine results in a reduction in ligand mediated mitogenesis, suggesting that c-Src association to this tyrosine is important in

PDGF mediated cell function. Therefore, this further suggests that the interaction between c-ErbB2/Neu and c-Src is critical for the tyrosine phosphorylation of the activation loop and the general activity of the receptor.

If one of the roles of c-Src is to facilitate the activation of the receptor, what is the necessity of associating with the receptor itself? The generation of the receptor complex provides a general platform for the nucleation and initiation of signaling from the surface. With receptor oligomerization, many directions in signaling can occur, one of which may be instigated by c-Src. A more direct role for c-Src in receptor function may actually involve the sustained activation of the receptor due to its direct interaction with c-ErbB2/Neu. Specifically, the association of c-Src to the activation loop tyrosine on c-ErbB2/Neu may prolong the open conformation of the kinase thereby extending receptor activity by protecting this site from the action of phosphatases.

3.3.3 *Differences in MAPK activation downstream from EGFR and EGFR^{YHAD}*

Interestingly, a difference is observed in the magnitude, duration and temporal activation of MAPK upon stimulation with EGF between the wild type EGFR versus TK and EGFR^{YHAD}. Potentially, the differences in MAPK activity may serve as an initial divergence point in signaling, resulting in the generation of different biological outputs. The conservative change in sequence between the EGFR and EGFR^{YHAD} that results in a clear difference in MAPK phosphorylation implies that a subtle change in receptor activation and catalytic activity can translate into differences in downstream signaling.

How can receptor activation generate specificity when common signaling molecules are utilized? A much-studied area in signal transduction is the mechanism of specificity mediated by the Ras/MAPK pathway. It is clear in the PC12 cell system that specific ligands can generate differences in MAPK activity resulting in differentiation and neurite outgrowth, or cellular proliferation. Specifically, differentiation in the presence of nerve growth factor (NGF) correlates with a sustained increase in MAPK activity in contrast to a transient increase in MAPK activity when cellular proliferation is initiated in the presence of EGF (Greene and Tischler, 1976; Huff et al., 1981; Traverse

et al., 1992). These differences have been suggested to attribute to the biological outcomes seen in PC12 cells upon stimulation with either NGF or EGF. Studies utilizing yeast show that the regulation of the yeast mating or growth pathways is dependent on the ability of the MAPK proteins Kss1 and Fus3 to perform specialized functions. Specifically, it was hypothesized that the presence of Kss1 drives yeast into the mating pathway only when Fus3 is absent. However recently it was found that Fus3 instead of mediating specificity by exclusion, modulates Kss1 activation directly by downregulating its activity. Thus signal specificity is mediated by limiting Kss1 function through phosphorylation via Fus3 (Sabbagh et al., 2001).

Within the PC12 cell system, the overt overexpression of receptors can in effect produce high levels of catalytic activity that can consistently drive a differentiation pathway, correlating with a sustained MAPK activation profile. For example, while PC12 cells do not express any endogenous PDGFR, its overexpression leads to sustained MAPK activation and differentiation that is PDGF dependent (Heasley and Johnson, 1992). Similarly, while endogenous levels of the insulin receptor do not activate MAPK and differentiation (Ohmichi et al., 1993), overexpression of the insulin receptor can initiate sustained activation of the MAPK pathway and induce differentiation (Dikic et al., 1994). In these examples however, it has not been shown as to what the consequences would be if an inducibly transient signal could drive a proliferation pathway. Nevertheless, this suggests that receptor function is closely linked in defining a biological response mediated by MAPK. In all likelihood, both receptor activation and MAPK activation can modulate specificity in biological responses.

Alternatively, it is possible that specificity may manifest itself via direct pathways downstream of the receptor. For example, it has been documented that a specific 80kDa protein called SNT is differentially expressed depending on whether the cell is undergoing proliferation or differentiation (Rabin et al., 1993). In addition, other downstream differences include the use of either Grb2 or Shc for EGF or NGF mediated signaling respectively (Buday and Downward, 1993a; Buday and Downward, 1993b; Stephens et al., 1994). Given this fact, it may be possible that the change within the

catalytic region to include the c-ErbB2 consensus has not only affected the activation profile of MAPK, but has altered the constellation of proteins that are found at the receptor complex. Specifically, since this region correlates with c-Src association it is possible that this difference, like the differences between the EGFR and the TrkA receptor in coupling differentially to Grb2 and Shc respectively, will mediate the difference in MAPK activation. Furthermore, evidence suggests that both Ras and c-Src interact with and are dependent on each other, for example dominant negative Ras (Stacey et al., 1991), neutralizing antibodies against Ras (Smith et al., 1986) and the introduction of p190RasGAP (DeClue et al., 1991) can suppress v-Src induced transformation. While the wild type EGFR, TK, and $EGFR^{YHAD}$ receptors display a similar phosphorylation pattern, only the TK and $EGFR^{YHAD}$ receptors associate with c-Src. Moreover, the biphasic MAPK response correlates with the two receptors that associate with c-Src suggesting that c-Src may play a role in mediating the downstream difference.

3.3.4 *Biological role of the EGFR, TK and $EGFR^{YHAD}$ receptors*

The above biochemical data suggests a difference in receptor signal transduction that is dependent on the presence of the c-ErbB2 kinase region. Furthermore, subtle differences that correlate with the association of c-Src to c-ErbB2/EGFR appear to alter the signaling capacity of the receptor in the presence of EGF. This suggests that differences in kinase regions and moreso, the presence of c-Src, may translate to a biologically significant outcome. To this end, it was found that indeed differences do exist if either the kinase region or the c-Src binding site is present, in this case within the context of the EGFR. Mammary gland related structures as assessed by mammosphere development suggest that early differences in alveolar-like structures correlate with the presence of the EGFR, TK or $EGFR^{YHAD}$ receptors. Consistent with the role of this receptor family in mammary gland development, previous reports demonstrate that wild type c-ErbB2 in EpH4 cells clearly results in alveolar formation that is dependent on both the MAPK and PI3'K pathways (Niemann et al., 1998). Interestingly, the fact that

temporal differences in MAPK activation are seen between the EGFR, TK or $EGFR^{YHAD}$, may point to a mechanism that defines the differential ability to form mammospheres. Furthermore, chimeric experiments that incorporated the kinase region of c-ErbB2 in the context of the Trk receptor (Niemann et al., 1998) provided a similar alveolar phenotype as seen with our TK chimeric receptor. Interestingly, the development of alveolar structures in EpH4 cells by TK can be reproduced with the presence of the altered site engineered in the $EGFR^{YHAD}$ construct. This subtle mutation has been found to correlate with the association of the c-Src kinase, further suggesting that c-Src may play a significant role in mammary gland development. However it is possible that the development of mammary gland structures mediated by the $EGFR^{YHAD}$ receptor may also involve other proteins that could differentially associate with this receptor. Furthermore, the difference in mammospheric development may not involve the specific association of proteins and instead mediates its phenotypic effects autonomously via its kinase activity.

Early experiments show inherent differences within the EGFR family with regards to mitogenesis and transformation. Indeed, c-ErbB2 appears to display a higher mitogenic and transformation potential when compared to the EGFR in fibroblasts (Di Fiore et al., 1987a; Di Fiore et al., 1987b). However, within the kinase region we find that c-Src may play a role in this, given that preliminary data suggests that YHAD can also mimic the mitogenic index of TK (Kim and Muller, unpublished results). Does c-Src play such a large role in mediating mitogenic potential? In NIH/3T3 cells it is required for the transit from S to G2 phase of the cell cycle (Roche et al., 1995a). Many studies with different receptor systems using chemical and dominant negative studies corroborate the fact that c-Src is important in promoting DNA synthesis. It may be that the coupling of c-Src to c-ErbB2, similar to other receptor systems, may play an important role in receptor activation and mitogenic potential.

3.3.5 *The role of c-Src in receptor activation and transformation*

The significance of c-Src in transformation has been addressed in a number of studies Most significantly, the ability of c-Src to severely attenuate MMTV/polyomavirus

mT mediated transformation within the mammary gland suggests that c-Src may play a critical role in mammary gland tumorigenesis (Guy et al., 1994a). Consistent with the above observations, we have shown that c-Src is important in Neu mediated transformation. In the presence of dominant-negative c-Src (Src251), Neu mediated transformation is severely impaired. This decrease in transformation ability is not an intrinsic defect in the cell lines that express Src251 since RasV12 can efficiently transform this cell line. Furthermore, the presence of Src251 can suppress Neu tyrosine phosphorylation, correlating with the ability of Src251 to suppress transformation. Therefore, consistent with other receptor systems where c-Src appears to play a role in PDGFR and CSF-1R signaling (Mori et al., 1993; Roche et al., 1995b; Twamley-Stein et al., 1993), c-Src also appears to play a significant role in Neu mediated signal transduction and transformation.

Previous evidence has suggested that c-Src may play a role in Neu mediated transformation. Indeed, the overexpression of the Neu RTK leads to an increase in c-Src kinase activity, correlating with its direct and specific association to the receptor itself. While the current evidence also suggests that c-Src associates with the activation loop of the receptor, what activates Src within this complex? Currently the answer is unclear, however the ability to activate c-Src may lie in the constellation of molecules found at the cell surface. Previous evidence points to receptor complexes that most likely contain not only c-ErbB2 clusters but also the EGFR and c-ErbB4. Indeed, receptor clustering has generally been speculated to prefer heteromerization rather than the generation of homomeric receptor complexes (Graus-Porta et al., 1997; Karunagaran et al., 1996). Thus it may be that the EGFR (Tice et al., 1999) or c-ErbB4 may by location and proximity activate c-Src. Another possibility may stem from the fact that many of the proteins associated with c-Src are cytoskeletal and are involved in cell adhesion and motility. For example, the integrin receptor system is found to be colocalized with c-Src along with the EGFR family thus creating a complex that is regulated by focal adhesion (Moro et al., 1998; Plopper et al., 1995). Cytoskeletal kinases found within focal adhesions such as Fyn, Abl and focal adhesion kinase (FAK) may participate in the activation of c-Src

(Schlaepfer et al., 1997; Schlaepfer et al., 1998). Indeed, the activation of FAK via integrin association results in the generation of a high affinity binding site specific for c-Src, potentially activating c-Src via substrate proximity (Schaller et al., 1994). The association of c-Src to FAK is required for the subsequent activation of the Ras pathway via Grb2 (Schlaepfer et al., 1997; Schlaepfer et al., 1994; Schlaepfer and Hunter, 1997; Schlaepfer et al., 1998) suggesting the importance of c-Src in focal adhesion signaling. Thus many mechanisms can influence c-Src activation and the subsequent activation of associated receptor tyrosine kinases.

The above data provides a potential role for Src in modulating receptor function upon activation. Here we show that Src has the ability to associate with a specific tyrosine, Y882, found within the activation loop of the kinase region of the receptor. The phosphorylation of this tyrosine, as observed in other receptor systems, appears to be critical for the catalytic activity of c-ErbB2. Our observations suggest that full catalytic activity of c-ErbB2 requires the phosphorylation of Y882 which then generates an open conformation of the activation loop thus permitting ATP into the binding pocket. The kinase that appears to provide the key molecular event to potentiate full receptor activation is c-Src. This now provides a potential explanation as to the reason why tumors induced by Neu display an increase in c-Src kinase activity as well as a direct association between the receptor and c-Src (Muthuswamy and Muller, 1995; Muthuswamy et al., 1994). Previous tumor models that utilize the Neu RTK to induce mammary gland tumorigenesis, these receptors are activated by various mechanisms based on constitutive dimerization. It is possible that their full activation and transforming potential hinges on the direct association of c-Src to the receptor, their functions being to phosphorylate the activation loops of the receptor and permit high levels of ATP loading. Indeed, we have shown that dominant negative Src appears to significantly suppress transformation mediated by activated Neu. Consistent with the role for c-Src in receptor activation, the presence of dominant negative Src results in a NeuNT receptor that is hypophosphorylated further suggesting the importance of c-Src in Neu mediated transformation. Furthermore, transformation mediated by activated Neu is severely

compromised when Y882 is converted to phenylalanine. With regards to specificity, the ability to generate specific tumor types with the overexpression of receptors such as c-ErbB2 and the EGFR may again reflect the specific constellation of cytoplasmic proteins associated with each receptor. The relative levels of such proteins like c-Src may contribute to tumor type specificity and ultimately the activation of specific downstream pathways.

3.3.6 *Chapter summary*

Previous data suggests that within the EGFR family, individual receptors can display differences in their ability to associate with cytoplasmic partners, potentially translating into distinct biological consequences. In particular, Neu induced tumors have been shown to display an increase in c-Src kinase activity that correlates with its direct association with the Neu receptor. Moreover, it was found that this association was specific to Neu and not to the closely related family member, the EGFR. In this section I have presented data that suggests that the direct and specific interaction of c-Src to the c-ErbB2/Neu receptor is unique in that it does not associate with the major phosphorylation sites that are found within the carboxyl region of the receptor. Instead, physical mapping utilizing site directed mutageneis and chimeric receptors have found that c-Src associates with the carboxyl terminal region of the kinase domain. Specifically, Y882 of the Neu receptor appears to be able to mediate c-Src association. Altering the binding site on the EGFR to the c-ErbB2 receptor consensus sequence found around Y882 not only restores the association of c-Src to the EGFR, it also alters the receptors phosphorylation profile upon EGF stimulation. Furthermore, the TK and $EGFR^{YHAD}$ receptors display differences in their ability to activate MAPK when compared to wild type EGFR, suggesting that the c-ErbB2 kinase region which correlates with the association of c-Src, can affect both receptor activation as well as the activation of MAPK. With regards to transformation potential, a dominant negative Src molecule was found to be able to suppress Neu mediated transformation that correlated with a decrease in receptor phosphorylation suggesting that c-Src plays a critical role in the activation of the receptor.

Receptor activation and the initiation of signal transduction are speculated to involve the stabilization of the receptor complex by a number of mechanisms. Ligand binding to the receptor has been the most widely believed mechanism in initiating receptor complex formation, however the presence of phosphatases and kinases that modify the receptor itself has been suggested to also play a role in receptor activation. These modifications may target critical residues that are found within the kinase region of the receptor. Specifically, the FGFR as well as the IR requires the phosphorylation of tyrosine residues that are found within the activation loop, their phosphorylation correlating with the activation of the receptor. Given the fact that c-Src can suppress c-ErbB2/Neu mediated transformation, it is possible that c-Src may play a direct role in receptor activation in conjunction with ligand binding and receptor clustering. It is believed that upon ligand association, receptor clustering can induce the activation loop to preferentially take on an open conformation, allowing the transphosphorylation of the critical tyrosines found within the region leading to the stabilization of catalytic activity. My data suggests that c-Src may also play a role in the stabilization of the kinase, thereby leading to receptor activation. While this may be the case, evidence that would indicate that c-Src directly phosphorylates the receptor is lacking. In order to address this, peptides to Y882 incubated with purified c-Src may address the direct relationship between the activation loop and c-Src.

While the analysis has attempted to be systematic in order to generate the binding site for c-Src to the c-ErbB2/Neu receptor, a caveat still exists. While the analysis of the tyrosines have focused on each as an independent signaling entity, it is still possible that c-Src may associate with more than one site at a time, a possibility that the above analysis could not address. It may have been possible to generate all permutations of null tyrosine sites, as attempted with the NYPD construct, however the ablation of all tyrosines on the receptor may compromise receptor function. In order to address the possibility of multiple sites of interaction between c-Src and c-ErbB2/Neu, one may utilize the technique from Rotin et al. (Rotin et al., 1992) to map interactions of the SrcSH2 domain to the c-ErbB2 receptor. In the presence of phosphatases, the SrcSH2 domain can protect

the tyrosine(s) from being dephosphorylated, these protected tyrosines can subsequently be identified. Indeed, this method was used in order to map the PLCγ binding site to the PDGFR (Rotin et al., 1992). Alternatively, one may address the problem by using peptides for each tyrosine found on the receptor, and then confirming the *in vitro* association with *in vivo* mutagenesis.

Differences in c-Src association appear to exist between the EGFR, TK and $EGFR^{YHAD}$ receptors, correlating with differences in MAPK activation, however it is still unclear as to whether any biological consequences exist between these receptors. In addition, it would be interesting to see whether any other differences in downstream signaling exist between the EGFR, TK and $EGFR^{YHAD}$ receptors. One avenue that may address this question is the generation of knock-in mice that express the receptor in question at its endogenous loci. This would be one direct way to address whether these subtle mutations within the EGFR family translate into differences in biology. However, given the subtle differences between these receptors it is very possible that the resulting mice may be phenotypically identical and without any overt physical problems. Granted this may be the case, differences at the molecular level may exist between the EGF receptors. With the advent of array technology, one can screen many target genes in order to see if any are differentially and subtly expressed.

While the preliminary observations suggest the importance of c-Src in Neu mediated transformation, other experiments to support this include the use of antisense, specific chemical inhibitors of c-Src, specific phosphorylated peptides to the site of interest or the use of inhibiting antibodies via microinjection to block c-Src activity. A relatively new technology first tested in *C. elegans* and in *Drosophila* involves an approach called RNA interference (RNAi) that can suppress specific mRNA transcripts from being transcribed, targeting them instead for destruction (Marx, 2000). While unsuccessful in mammalian cells, recently a breakthrough occurred in that shorter versions of RNAi, called small interfering RNA (siRNA) can when microinjected, efficiently suppress specific genes from being expressed in mammalian cells (Elbashir et al., 2001). It may be possible to investigate whether siRNAs could phenotypically revert

NeuNT transformed cells back to an untransformed state. This 'knock-down' approach could potentially avoid the ill success associated with antisense or the lack of specificity that could be associated with inhibiting antibodies or chemicals.

The obvious experiment is to use c-Src null cell lines that harbor an activated form of Neu, however this was proven to be problematic. Firstly, it appears that specific clonal populations isolated from c-Src null embryonic fibroblasts display variability in its ability to support focus formation mediated by NeuNT (data not shown). This was puzzling at first but not unprecedented since in the PDGF receptor system where c-Src has been found to be in all experimental cases critical in PDGF mediated signal transduction, stimulation with PDGF in an embryonic fibroblast cell line that has all ubiquitous Src family members ablated (SYF/c-Src, Yes, Fyn) apparently has no problem activating the PDGFR and stimulating DNA synthesis (Klinghoffer et al., 1999). The conflict in observations may reside in the fact that all embryonic fibroblasts derived from these null mice have been immortalized by SV40 large T antigen. Large T antigen has been suspected to immortalize cells through its association with the tumor suppressors Rb and p53 (DeCaprio et al., 1988; Levine and Momand, 1990). Indeed, recent studies have suggested that large T can subvert even cell lines that express dominant negative (inhibiting) versions of Ras and Src (Broome and Courtneidge, 2000). Given this possibility, results obtained from c-Src null cell lines should be interpreted with caution. Nevertheless, the Src null cell line experiments may provide valuable data concerning the role of c-Src in Neu mediated transformation.

CHAPTER 4

Normal Mammary Gland Development Requires a Functional c-Src Protein Tyrosine Kinase

4.1 Introduction

The initiation and progression of human breast cancer is now known to involve a complex interplay of genetic, biochemical, and hormonal factors that are tightly regulated in normal proliferating cells. However, when unchecked these same factors can potentially lead to transformation and tumorigenesis. Ultimately, cell populations that have undergone detrimental mutations can acquire and display characteristics such as positive feedback, the suppression of negative feedback as well as apoptosis, angiogenic potential, sustained replicative potential, and tissue invasive capabilities (Hanahan and Weinberg, 2000). Cell populations of this nature have a definite selective growth advantage that in many cases leads to oncogenesis. One mandate in cancer research is to identify consistent molecular markers that can predict the severity of the disease and correlate this with the prognosis of the patient. Indeed, the c-*erbB2/neu* receptor tyrosine kinase has been found to fit the above criteria both in the clinic as well as in animal models (Andrechek et al., 2000; Andrulis et al., 1998; Gullick et al., 1991; Guy et al., 1996; Muller et al., 1988; Siegel et al., 1994; Slamon et al., 1987; Slamon et al., 1989).

In addition to growth factor receptors, hormone receptors such as the estrogen receptor are intimately linked to breast cancer progression. As with *erbB2*, the estrogen receptor also appears to be an accurate prognostic indicator of disease severity. In this scenario, a poor patient prognosis correlates with the loss of estrogen receptor function. Indeed, with current breast cancer therapies heavily dependent on adjuvant anti-estrogens (Osborne, 1998), it is not surprising that a significant increase in morbidity and mortality occurs with the development of tamoxifen resistance (Osborne and Fuqua, 1994; Wiebe et al., 1993). Interestingly, the overexpression of *erbB2* observed in breast cancer patients

also significantly correlates with tamoxifen resistance (Carlomagno et al., 1996; De Placido et al., 1998; Grunt et al., 1995; Gullick et al., 1991; Houston et al., 1999), suggesting that surface and nuclear receptor crosstalk may promote the conversion to an aggressive tumor phenotype.

An extensive body of literature exists regarding the importance of estrogen receptor function both in breast cancer and in the promotion of normal mammary epithelial proliferation. Alveolar formation and ductal branching is a process highly dependent on multiple cues directing its development. One of the predominant regulators of this process is 17β-estradiol (E2) and its receptors, estrogen receptor α and β (ERα and β), are members of a superfamily of nuclear steroid receptors that share common functional domains. The biological importance of ERα was revealed with the generation of the estrogen receptor α knockout (αERKO) mouse. Mammary gland, ovarian and uterine development within the αERKO mice displayed dramatic developmental defects, supporting strongly the importance of the ER in reproductive development. Female αERKO mice are infertile and possess hyperemic ovaries that harbor primary and secondary follicles that do not progress to the antral stage. Large atretic haemorrhagic cysts within these ovaries suggests that a block in folliculogenesis occurs prior to the antrum stage (Korach et al., 1996; Lubahn et al., 1993). Mammary glands from αERKO mice display vestigial ducts that do not fully develop regardless of circulating estradiol levels. Taken together, these observations suggest that ERα plays a distinct physiological role in estrogen mediated signaling.

While the molecular mechanism is currently poorly understood, the biochemical and biological mechanism of ERα is slowly being elucidated. The current dogma involves the association of estradiol to the estrogen receptor inducing its dimerization, thereby generating an active transactivation complex that is capable to bind estrogen responsive element (ERE) containing genes. Although simple on first observation, this pathway is now known to be substantially more complex. Genetic and biochemical analyses of ER function have revealed that transactivation is mediated by two domains termed AF1 and AF2. The AF1 region confers constitutive and ligand-independent

transactivation whereas AF2 function is dependent on hormone binding (Berry et al., 1990). The constitutive activation of the ER by AF1 is found to further correlate with the phosphorylation of specific residues found within this region. Indeed, serine 118 has been shown to be the specific target of mitogen activated protein kinase (MAPK) (Kato et al., 1995). Its importance has been demonstrated in that the ability of MAPK to phosphorylate serine 118 within the AF1 region is sufficient in mediating estrogen receptor activation. This further suggests that cytoplasmic factors have the ability to regulate estrogen receptor function.

Recent evidence suggests that the c-Src protein tyrosine kinase may play an important role in ER activation. For example, in cell culture experiments the activation of ERα correlates with an increase in c-Src kinase activity (Di Domenico et al., 1996; Migliaccio et al., 1993), this activity when blocked using specific c-Src inhibitors can inhibit estrogen mediated ER activation (Di Domenico et al., 1996; Migliaccio et al., 1993; Migliaccio et al., 1998). Furthermore, upon dephosphorylation of ERα, the introduction of Src induces the tyrosine phosphorylation of ERα and subsequently rescues ERE binding. Given the association between ERα and c-Src, estrogen mediated activation of MAPK can thus be speculated to involve the phosphorylation of the adaptor protein Shc leading to its association to Grb2 and the activation of Ras. Indeed in tissue culture, the activation of Ras correlates with Shc phosphorylation. Furthermore, phosphorylation of serine 118 by MAPK on ERα activates it in a ligand independent fashion and promotes nuclear translocation, suggesting that both c-Src and the MAPKs are important in promoting the activation of the ER.

Given the mammary gland phenotype of the αERKO mouse, in conjunction with the role of c-Src in ER activation, the above data suggests that c-Src may play an important role in ERα mediated mammary gland development. Whether c-Src plays a role in the development of other reproductive organs is unclear. Furthermore, biological data that correlates a role for c-Src in ERα mediated reproductive development *in vivo* is lacking.

4.2 Results

4.2.1 c-Src is critical for normal ductal morphogenesis

The importance of the ER in mammary gland development has been established both *in vitro* utilizing cell culture systems and most convincingly *in vivo* with the establishment of the αERKO mouse. However, the potential biological role of c-Src in mammary gland development as well as its involvement with the ER is unclear. Given the above evidence, one prediction would be that gross morphological differences would exist between mammary glands derived from c-*src* null mice versus c-*src* wild type mice. Consistent with this hypothesis, a dramatic defect is observed in the development of the ductal tree in c-*src* null mice (Figure 4.1). At each time point from 3 to 10 week-old virgin females, the development of the mammary tree is severely retarded. This does not appear to be due to the runted nature of the c-*src* null phenotype since at 6-10 weeks of age c-*src* null mice are equivalent in size when compared to their wild type littermates (data not shown). Furthermore, the size of the c-*src* null fat pad although smaller than the wild-type from 4-6 weeks of age do become comparable in size to wild type littermates beyond 6 weeks of age (data not shown), suggesting that the observed difference in ductal outgrowth is not due to the size of the mouse or its reduced fat pad.

Significantly, the number of terminal end buds (TEBs) in the c-*src* null mouse is significantly less than that found in the wild type mammary tree even up to 10 weeks. Using the centre of the lymph node as a landmark it is also observed that the defect in mammary tree growth is most significant at 6 weeks of age (Figure 4.2). Thus, the potential defect may lie in the inability to form sufficient numbers of TEBs early in development translating into a reduction in ductal outgrowth. Interestingly upon further analysis, at 20 weeks of age the architecture of the c-*src* null mammary tree appears comparable to wild type (data not shown) suggesting that other molecular mechanisms may compensate for the observed branching defect. Histological evaluation reveals no obvious abnormalities in ductal structure at each time point (Figure 4.3). The c-*src* null

Figure 4.1 c-Src null mice display a mammary gland defect.

Representative wholemount analysis of 3, 6 and 10 week old virgin female mammary glands (fourth inguinal) from Src wild type or Src null mice. Each age represented displays a defect in terminal end bud (TEB) formation as well as retarded outgrowth of the ductal tree. The lymph node (LN) serves as a convenient reference point to evaluate ductal outgrowth. All diagrams are at 6.4x magnification.

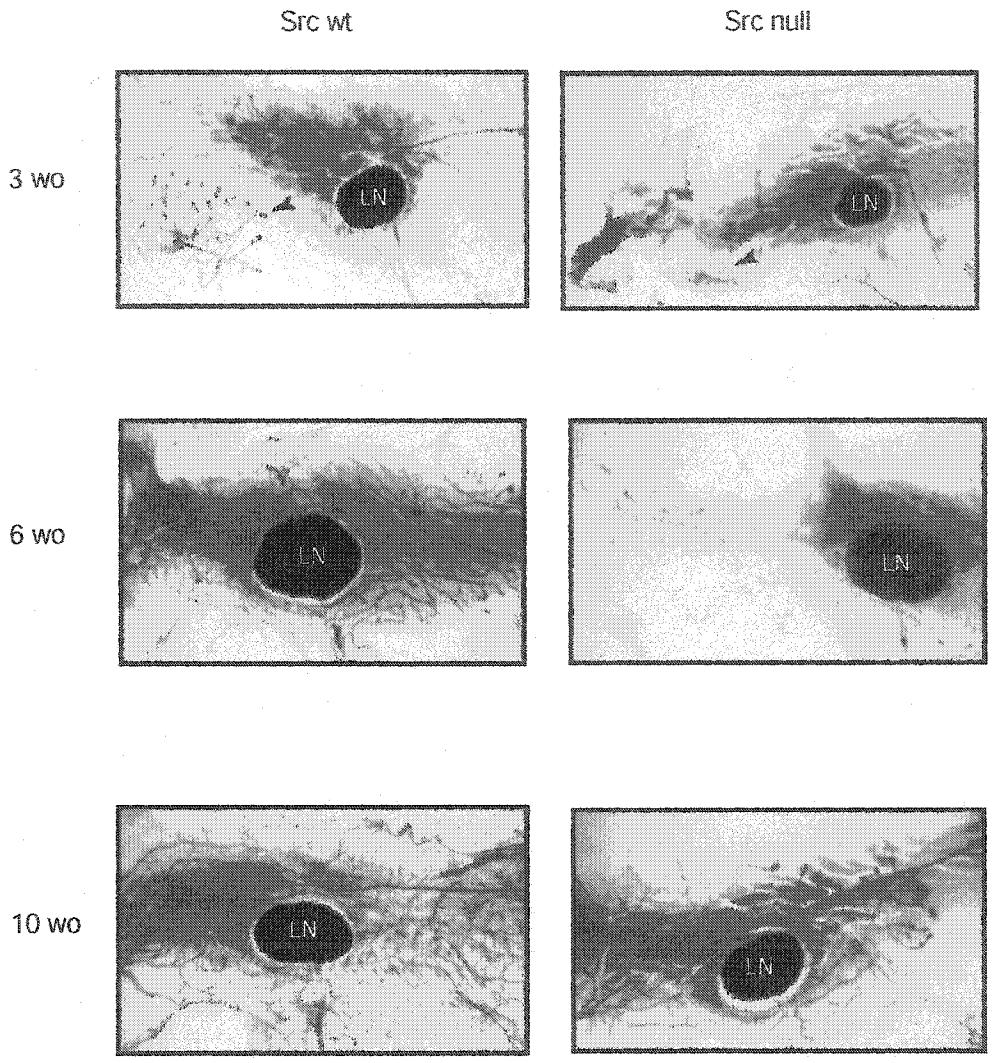
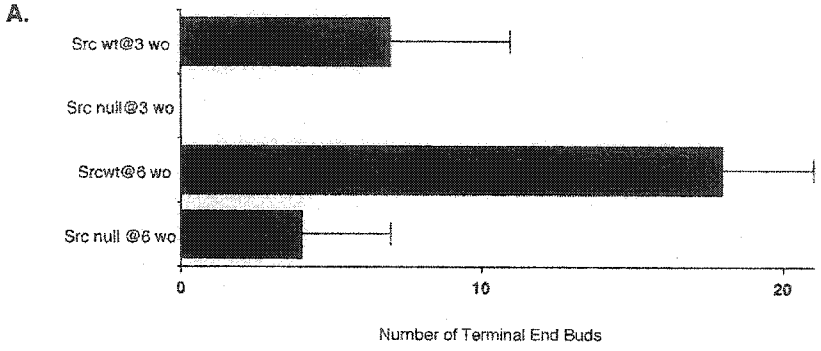

Src wt
Src null
3 wo
6 wo
10 wo
LN
LN
LN
LN
LN
LN

Figure 4.2 Quantitative analysis of terminal end bud formation as well as growth characteristics of mammary dutal outgrowth.

Wholemounts from two Src null mammary glands and their corresponding age matched wild type samples from 3, 6 and 10 weeks of age were analyzed. (A) Terminal end bud formation is significantly less in Src null virgin female mice when compared to an age matched c-*src* wild type mammary gland. (B) The centre of the lymph node can be used as a marker, the left side (minus) representing the direction towards the nipple and the right side (plus) representing the direction of ductal outgrowth. Although the distance from the leading edge of the ductal outgrowth to the centre of the lymph node in 3-week old mice is comparable, at 6 and 10 weeks of age growth is delayed significantly.


A.
Src wt@3 wo
Src null@3 wo
Srcwt@6 wo
Src null @6 wo
0
10
20
Number of Terminal End Buds

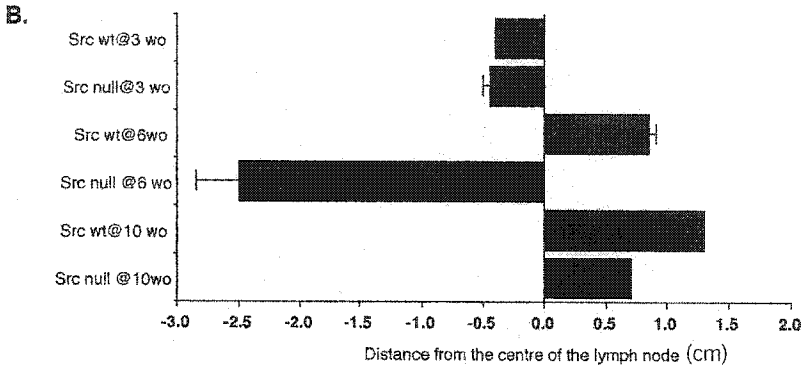
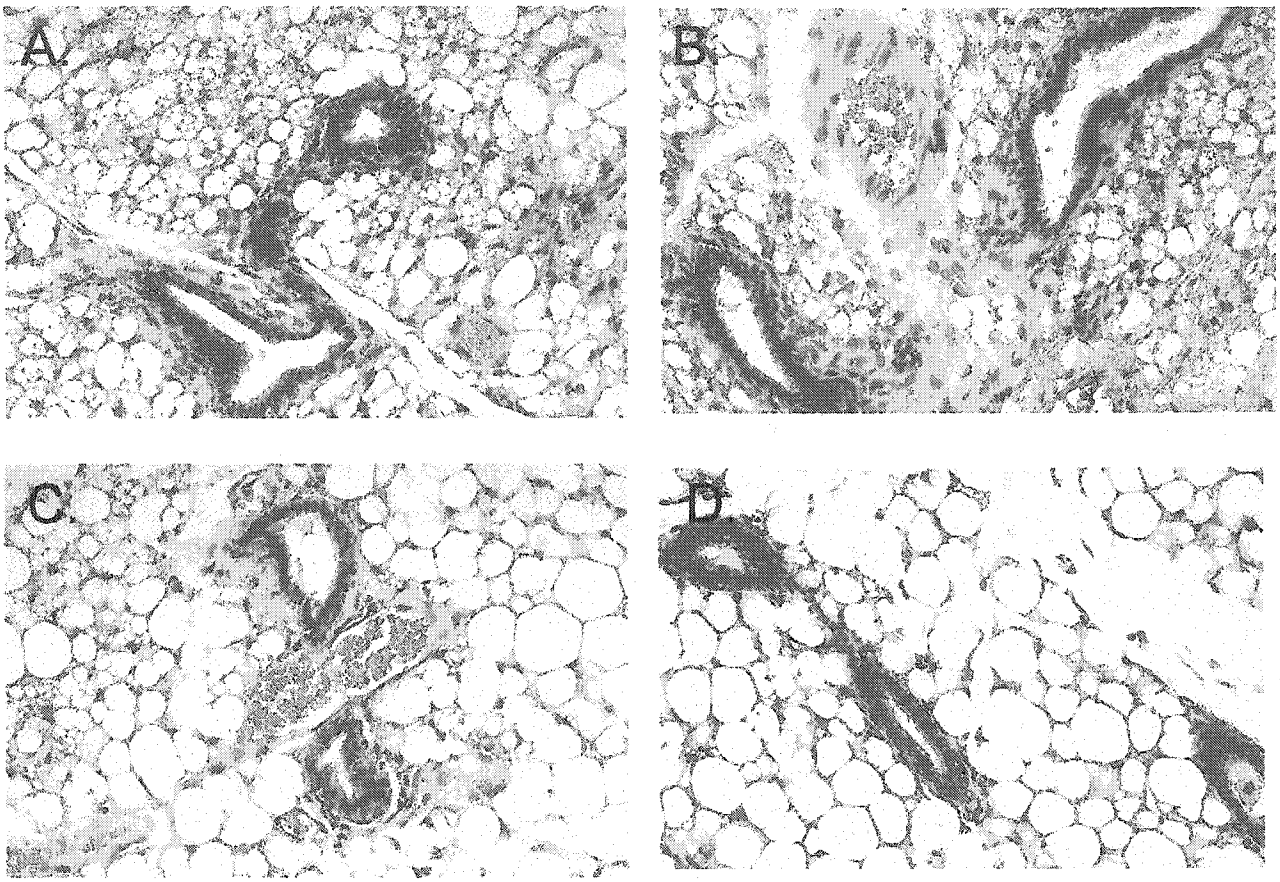

B.
Src wt@3 wo
Src null@3 wo
Src wt@6wo
Src null @6 wo
Src wt@10 wo
Src null @10wo
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
Distance from the centre of the lymph node (cm)

Figure 4.3 Histological evaluation of Src null mammary glands.

Sections of the inguinal gland (#4 mammary gland) of c-*src* null mammary glands and their age matched wild type controls at 3 (A and B), 6 (C and D) and 10 (E and F) weeks of age were embedded and stained with hematoxylin/eosin.


A
B
C
D

mice are fertile and lactate normally suggesting a fully functional mammary gland (Soriano et al., 1991).

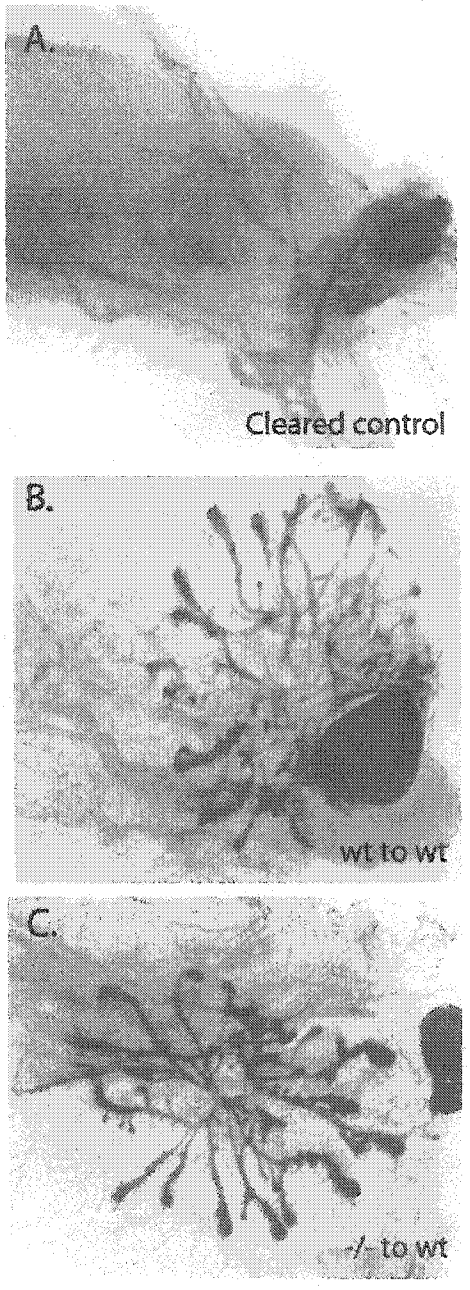
4.2.2 Tissue transplants suggest a stromal mammary gland defect in c-*src* null mice.

It is evident that estrogen plays a key role in mammary gland development, via direct stimulation of TEB development and cellular proliferation (Daniel et al., 1987). While the ERα has been localized to both the ductal epithelia and the surrounding stromal compartment (Daniel et al., 1987; Haslam and Nummy, 1992), current data suggests a compartmental difference in the functional role of the estrogen receptor in mammary gland development (Cunha, 1994; Cunha et al., 1997). However, estrogen localization within the cap cells of the TEBs display no expression, suggesting that the effects of estrogen are indirect (Haslam and Nummy, 1992; Zeps et al., 1998). Indeed, ERKOα mammary gland tissue transplanted into the cleared fat pad of wild type mice demonstrated that ERKOα mouse phenotype was a stromal defect.

Given the now apparent importance of stromal-epithelial communication within the mammary gland, a similar approach was undertaken in order to assess whether the mammary gland defect observed within the c-*src* null mice is due to the stromal or epithelial component of the c-*src* null mammary gland. If the defect is attributable to the epithelial component of the mammary gland, then transplanting null epithelia into a cleared wild type fat pad should result in a ductal outgrowth defect. Conversely, if the null defect is due to the stromal component, then transplant of null tissue into the wild type cleared fat pad should result in rescue and relatively normal ductal outgrowth. To this end, tissue from a c-*src* null virgin female mouse or a wild type control was excised by sharp dissection and transplanted into a cleared fat pad of a wild type 3 week-old syngenic virgin female mouse (Figure 4.4). Three weeks post-surgery the glands were excised and processed for wholemount analysis. Interestingly, no apparent difference in ductal outgrowth was observed when comparing wild type versus c-*src* null transplants at

Figure 4.4 Transplants from c-*src* null to a wild-type cleared mammary gland suggest a stromal defect.

The 4th inguinal gland of three-week-old virgin Fvb/n mice were cleared (A) and syngenic tissue from virgin female c-*src* null mice were transplanted. Whole mount analysis was performed 3 weeks post-surgery of wild-type controls (B) and c-*src* null (C) transplants. Magnification of 1.6x.


A.
Cleared control
B.
wt to wt
C.
-/- to wt

this stage suggesting that the c-*src* null mammary gland defect is a non-autonomous, stromal effect.

4.2.3 Uterine and ovarian phenotype revealed in c-src null mice

In addition to the mammary and ovarian phenotype displayed by the ERKOα mice, they also possess hypoplastic uteri that do not respond to E2. Analysis of uteri excised from wild type and c-*src* null mice show that they are grossly dissimilar at 3 and 6 weeks of age. While the uterus from c-*src* null mice appears hypoplastic (Figure 4.5), the epithelial, myometrial and stromal layers while reduced in size are intact. However, unlike the ERKOα mice where the uterus remains underdeveloped, c-*src* null uteri appear to gain weight and can support embryonic development.

While it appears that the loss of c-Src negatively affects ductal outgrowth, it is possible that the mammary gland phenotype may be due to factors that are independent of the mammary gland itself. Indeed, ERKOα mice not only display a mammary gland defect but also an ovarian phenotype in that oocytes fail to reach maturity and degenerate forming massive ovarian hemorrhagic cysts. In order to address the possibility that the loss of c-Src may also result in an ovarian phenotype, ovaries were removed from wild type and c-*src* null mice at four and 6 weeks of age (Figure 4.6). Histological analysis reveals that while wild type ovaries display oocytes at all stages of development from primary oocytes to mature late stage Graafian follicles, c-*src* null ovaries display only primary and secondary oocytes that do not display any late stage follicular development. However, c-*src* mice are fertile and do lactate, suggesting that like the mammary gland phenotype there is a general delay in the development of the reproductive system that correlates with the loss of the c-Src PTK.

4.2.4 Defect in ERα signaling in c-src null mammary gland explants

The phenotype of the c-*src* null mammary gland is clear, however the underlying molecular mechanism is unknown. Given the significance of c-Src in ERα signaling, one potential explanation for the mammary gland defect may reside in the inability of the

Figure 4.5 Uterine phenotype in c-*src* null mice.

(A) Gross morphology of postnatal reproductive tracts from wild-type and c-*src* null mice. Shown are the uterine structures from 4 and 6 week-old virgin female mice. (B) Histological examination by hemotoxylin/eosin of a cross-section from a 4 week-old uterus.

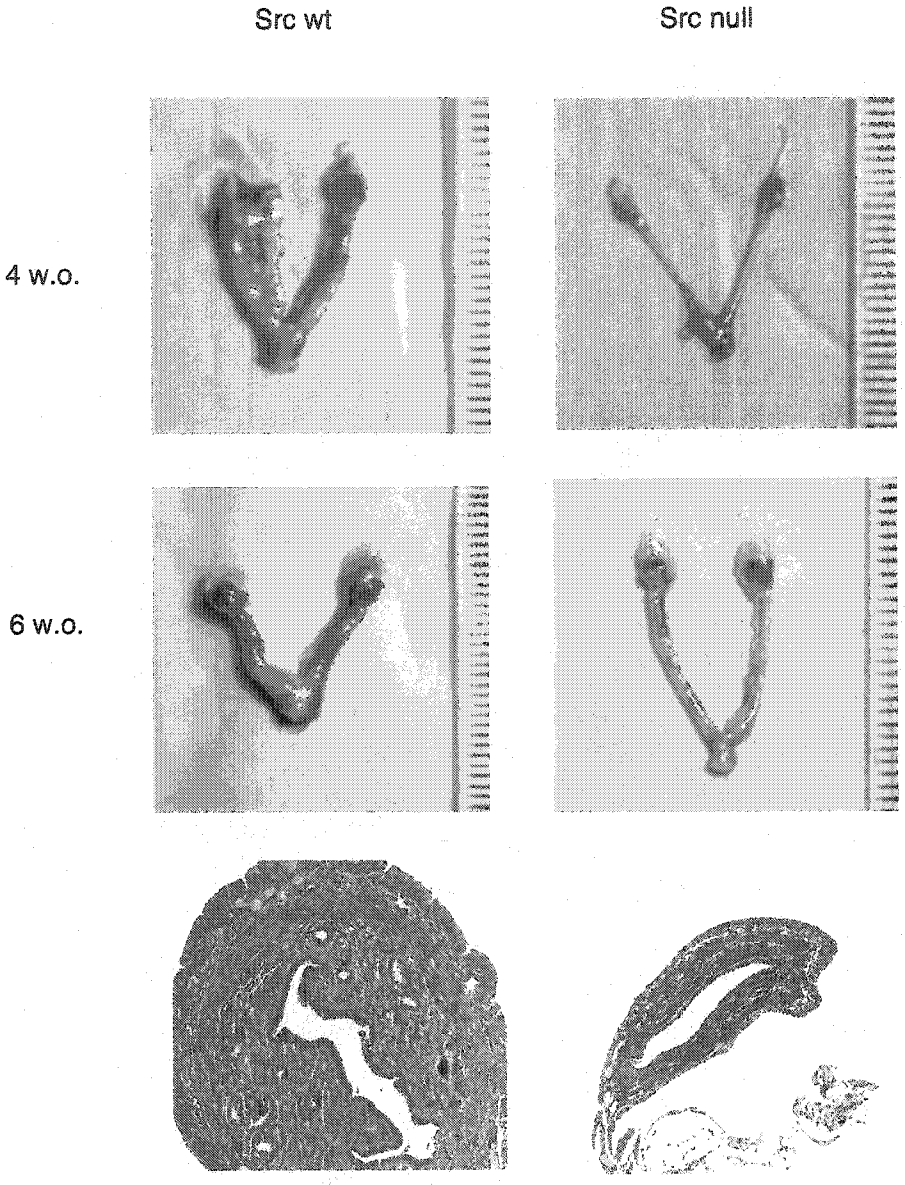
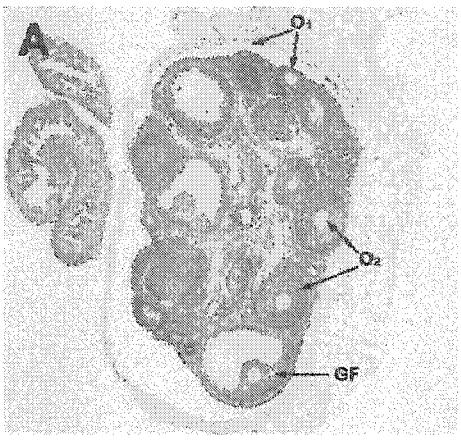
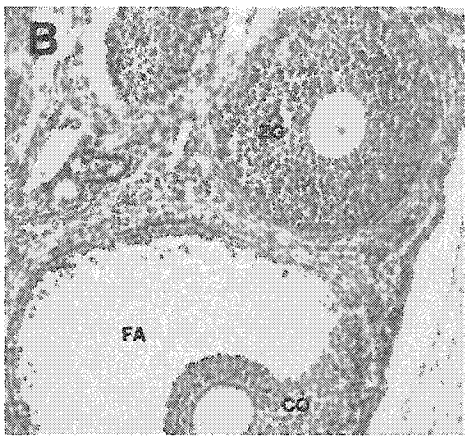
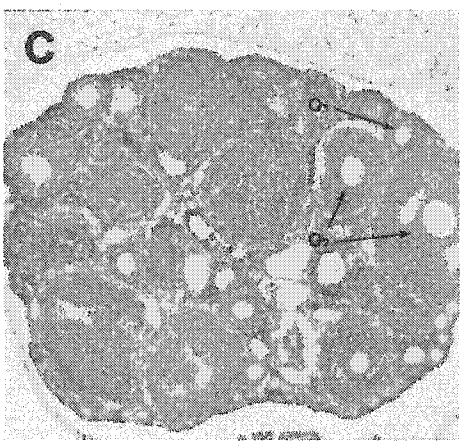

Src wt
Src null
4 w.o.
6 w.o.

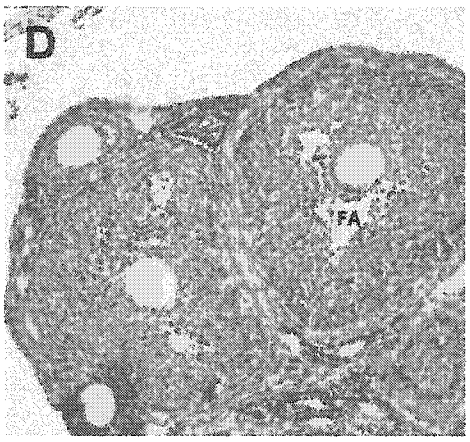
Figure 4.6 Ovarian phenotype in c-src null mice.

Ovaries were excised for histological examination from virgin wild type (A and B) and c-*src* null mice at 4 weeks of age at 50X (A&C) and 100X (B&D) magnification. Primary oocytes (O1), secondary oocytes (O2), mature Graafian follicles (GF), follicular antrum (FA), zona granulosa (ZG) and cumulus oophorus (CO) are indicated.


A
O1
O2
GF


B
SG
FA
CG


C
O1
O2


D
FA

ERα to propagate a signal upon estrogen stimulation. In order to address this possibility, mammary glands were excised from wild type and c-*src* null mice and their mammary epithelial cells (MECs) were cultivated with the intent to study the ERα in response to estrogen. Upon estrogen stimulation wild-type explants display an increase in ERα levels in association with its tyrosine phosphorylation (Figure 4.7 a and b). In contrast, explants derived from the null mammary gland fails to display detectable levels of ER upon estrogen stimulation. The observed difference in ER levels between wild type and null mammary glands are not due to differences in epithelial content between the two explants since similar levels of an epithelial specific marker are observed (Figure 4.7c). Consistent with the above observations, the loss of c-Src is found to negatively impact ER mRNA levels at multiple timepoints. Estrogen receptor transcript levels decrease an average of 1.6 fold over all timepoints in c-*src* null MECs (Figure 4.8; Table 4.1). This suggests that the loss of c-Src may impact negatively on ER levels within the mammary gland, which translates into the observed developmental defect.

To further suggest that a loss of c-Src can impact negatively on ER levels, MCF-7 cells were derived to express a dominant negative version of c-Src (Src251) (Figure 4.9). In contrast to wild type MCF7s, estrogen stimulation of MCF7:Src251 cells display a decrease in ER levels which also correlate with a low level of tyrosine phosphorylation. These results are consistent with the MEC explants suggesting that the presence of Src251 effect negatively the levels of the estrogen receptor.

4.2.5 Defect in MAPK activation upon E2 stimulation in c-src null mammary gland explants

One important consequence of the association between c-Src and ERα is the stimulation of the Ras-MAPK signaling pathway. To assess whether the MAPK pathway was adversely affected in c-*src* deficient mice, primary MEC explants from wild type and mutant *c-src* animals were stimulated with estradiol over time. Immunoblot analyses with c-Src specific antisera confirm that each cell population was derived from the appropriate genotype (Figure 4.10). To explore whether MAPK activity was affected in these strains,

Figure 4.7 ER phosphorylation is dependent on the presence of wild type Src.
(A) Mammary gland explants were cultured in charcoal stripped serum (CSS) and stimulated with 100nM E2 for 10 minutes. ERα was immunoprecipitated and immunoblotted with anti-ERα. (B) Additionally, the level of phosphotyrosine was detected by immunoprecipitating ERα and immunoblotting with anti-phosphotyrosine (PY20). (C) Epithelial content in wild type and c-Src null mammary gland explants. An epithelial keratin antibody that reacts broadly with epithelia (AE3) (Sorenson et al., 1987) was used as a measurement for epithelial content in lysates derived from wild type and c-src null mammary gland explants.

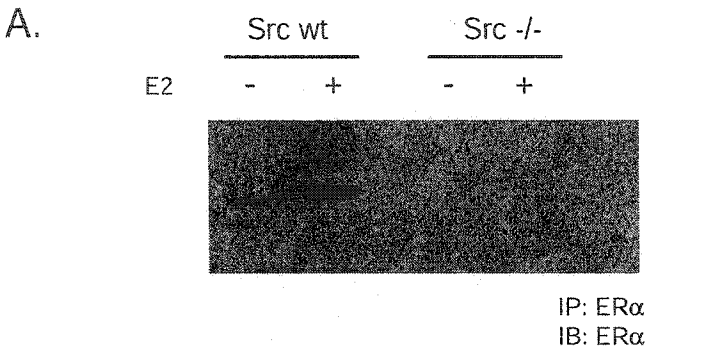

A.
Src wt
Src -/-
E2
-
+
-
+
IP: ERα
IB: ERα
B.
Src wt
Src -/-
E2
-
+
-
+
IP: ERα
IB: PTyr
C.
Src wt
Src -/-
E2
-
+
-
+
IB: Keratin AE3

Figure 4.8 ERα mRNA levels from wild type and c-*src* null MECs.

Mammary gland explants from wild type and c-*src* -/- mice were cultivated in CSS, serum starved and exposed to 100nM E2 over a defined timecourse. Total mRNA was isolated and levels were quantitated by Lightcycler using specific primers for ERα (see materials and methods). Results were normalized with results from specific primers for PGK and all numbers were calculated relative to wild-type RNA at $time_0$. Results are an average of two Lightcycler runs for all samples.

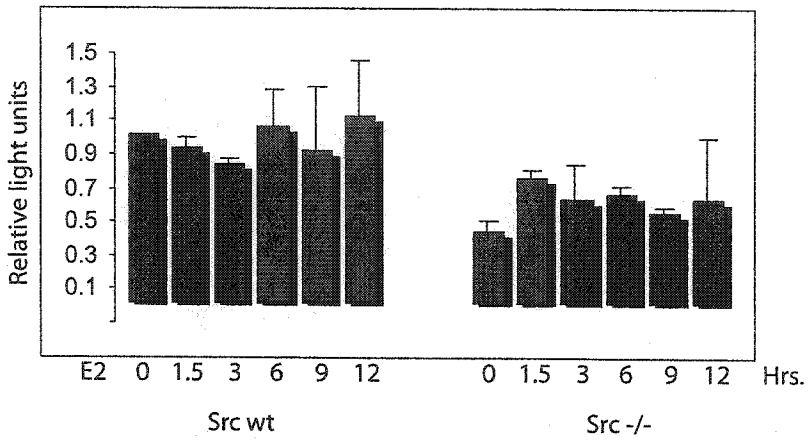

Relative light units
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
E2 0 1.5 3 6 9 12
0 1.5 3 6 9 12 Hrs.
Src wt
Src -/-

Table 4.1 Estrogen receptor RNA levels

			Src wt						Src null						
		E2	0	1.5	3	6	9	12	0	1.5	3	6	9	12	Hrs.
Run 1	**Estrogen receptor** a		4.72	3.70	3.86	4.02	2.66	4.03	2.28	3.50	2.05	2.70	2.71	3.57	
	PGK a		8.21	7.53	8.51	7.98	7.47	7.96	10.48	8.59	8.00	7.73	8.58	7.17	
	Ratio ER/PGK b		0.575	0.491	0.454	0.504	0.356	0.506	0.218	0.407	0.256	0.349	0.316	0.498	
	Normalized to wt@0 c		1.000	0.855	0.789	0.876	0.619	0.880	0.378	0.709	0.446	0.607	0.549	0.866	
Run 2	**Estrogen receptor** a		5.33	5.26	5.33	5.60	4.99	6.00	2.99	4.56	3.60	3.62	2.38	1.81	
	PGK a		6.57	6.75	7.66	5.67	5.18	5.41	7.39	7.29	5.52	6.42	5.54	6.53	
	Ratio ER/PGK b		0.811	0.779	0.696	0.988	0.963	1.109	0.405	0.626	0.652	0.564	0.430	0.277	
	Normalized to wt@0 c		1.000	0.961	0.858	1.218	1.188	1.368	0.499	0.771	0.804	0.695	0.530	0.342	
	Estrogen receptor		1.000	0.908	0.823	1.047	0.904	1.124	0.439	0.740	0.625	0.651	0.540	0.604	
	+/- stdev d		0.000	0.075	0.049	0.242	0.402	0.344	0.085	0.044	0.254	0.062	0.014	0.371	

a Numbers that represent the area under the specific peak for each amplification reaction.
Each area under the curve was read at least twice and the average calculated.

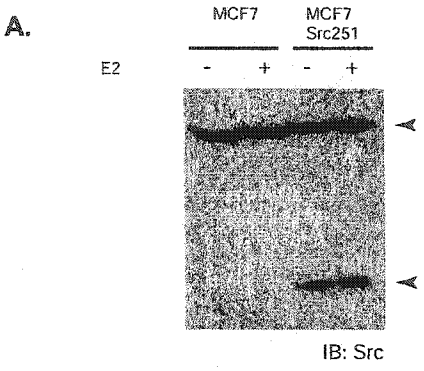
b Areas under the specific peak for each amplification reaction was normalized against the internal control PGK for each independent time point.

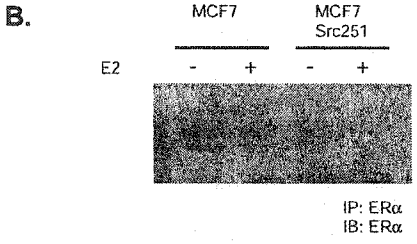
c All independent ratios for each timepoint was standardized relative to the ratio of wild type MECs at time 0.

d Average area under the specific peak for each amplification reaction normalized against PGK
and weighted to Src wt at time 0 +/- standard deviation.

Figure 4.9 Presence of a dominant negative c-Src negatively modulates the levels of ERα.

(A) MCF7 cells that express a dominant negative version of c-Src (Src251) were derived. (B) Parental MCF7 and MCF7Src251 mammary epithelial cells were stimulated with 100nM E2 for 10 minutes and ERa was immunoprecipitated from each. The levels of ERα was detected by immunoblotting with anti-ERa. (C) Additionally, the level of tyrosine phosphorylation was measured by immunoprecipitating with ERa and immunoblotting with anti-PTyr.


A.
MCF7
MCF7
Src251
E2
- + - +
IB: Src


B.
MCF7
MCF7
Src251
E2
- + - +
IP: ERα
IB: ERα

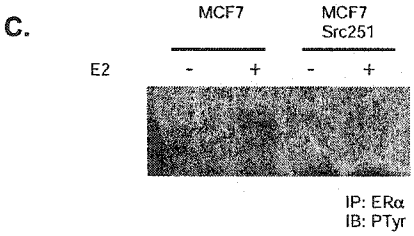
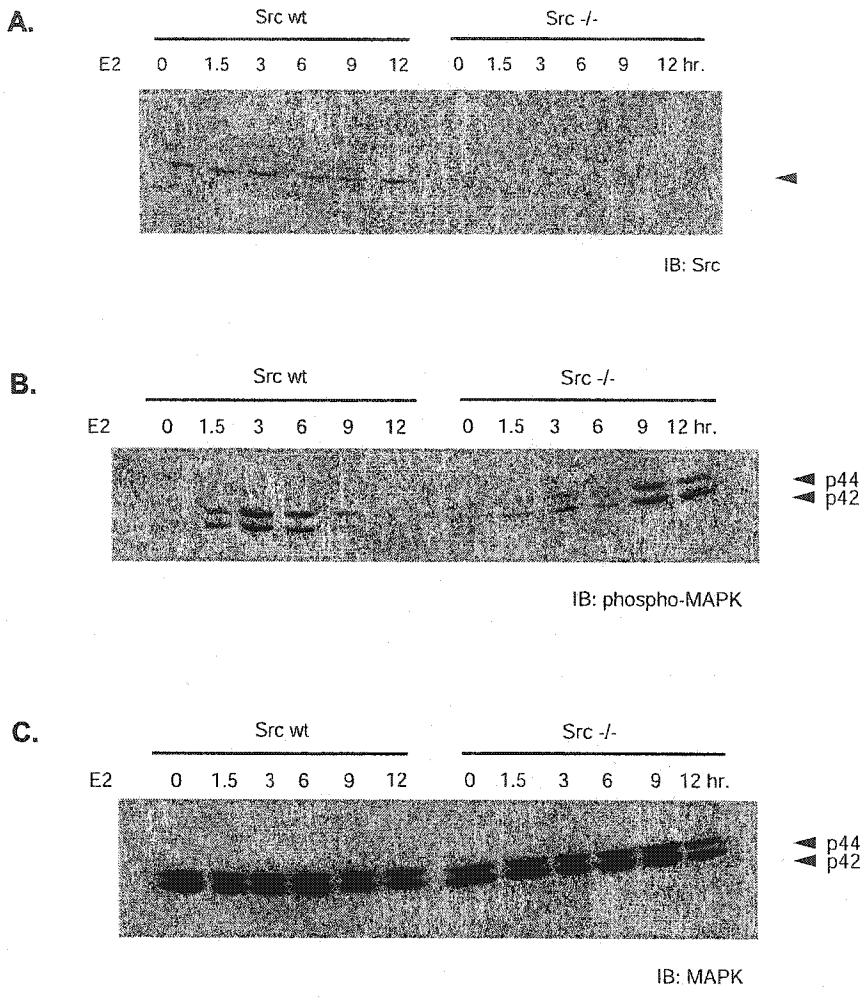

C.
MCF7
MCF7
Src251
E2
- + - +
IP: ERα
IB: PTyr

Figure 4.10 Absence of c-Src negatively impacts MAPK activation in the presence of E2.

(A) Mammary gland explants from wild type and c-*src* -/- mice were cultivated, serum starved and exposed to 100nM E2 over a defined timecourse. (B) Upon E2 stimulation for the defined timepoints lysates were collected and subjected to immunoblot analysis with phospho-MAPK. (C) The levels of activated MAPK was not due to differences in total MAPK.


A.
Src wt
Src -/-
E2 0 1.5 3 6 9 12 0 1.5 3 6 9 12 hr.
IB: Src
B.
Src wt
Src -/-
E2 0 1.5 3 6 9 12 0 1.5 3 6 9 12 hr.
p44
p42
IB: phospho-MAPK
C.
Src wt
Src -/-
E2 0 1.5 3 6 9 12 0 1.5 3 6 9 12 hr.
p44
p42
IB: MAPK

protein lysates from these cell cultures were subjected to immunoblot analyses with phosphospecific MAP kinase antibodies. The results reveal that in the absence of a functional c-Src, wild type MECs stimulated with estradiol results in an increase in MAPK activity that peaks at 3 hours post-stimulation and then decreases in a temporally dependent fashion. In contrast, stimulation of c-*src* deficient cells results in MAPK activation that peaks at 9 hours post-stimulation, when compared to wild type MECs. The difference in cellular response was not due to differences in MAPK protein suggesting that within MECs the loss of c-Src result in the inability to activate the MAPK pathway in the presence of estradiol.

While the above data suggests that estrogen can affect the activation of MAPK, it is unclear whether this is directly mediated. In order to address whether MAPK activation mediated by estrogen is specific, MECs from both wild type and c-*src* null mammary glands were treated with U0126, an inhibitor that directly and specifically effects the activity of MAP kinase kinase (MEK), an upstream activator of MAPK (Figure 4.11). While unstimulated cells at t_0 display a basal level of MAPK activity, all MAPK activity is suppressed in both wild type and null MECs upon treatment with U0126 suggesting that the effect observed to MAPK is estrogen dependent and specifically activates the MAPK pathway.

To further provide evidence that the loss of c-Src function negatively effects Ras signaling, MCF7:Src251 cell lines were analyzed to address whether a dominant negative version of c-Src can impact MAPK activation. Upon stimulation with estradiol we observe an inability to efficiently activate MAPK in the presence of Src251 when compared to wild type MCF7 cells (Figure 4.12). Indeed, while MAPK activity in the parental MCF7 cells peak at 10 minutes post-stimulation, MCF7 cells that harbor the dominant negative do not show such an increase in MAPK activity. The lack of activation is not due to differential levels of MAPK, further suggesting that the loss of c-Src function negatively impacts on MAPK signaling.

Figure 4.11 U0126 abrogates MAPK activation upon estrogen stimulation of wild-type and c-*src* null MECs.

Mammary gland explants from wild type and c-*src* -/- mice were cultivated, serum starved and exposed to 100nM E2 over a defined timecourse either in the absence or the presence of U0126, a MEK inhibitor, over the total time of stimulation with estrogen. (A) The ability to activate MAPK was assessed by anti-phosphoMAPK, (B) the differential phosphorylation was not due to differences in MAPK levels.

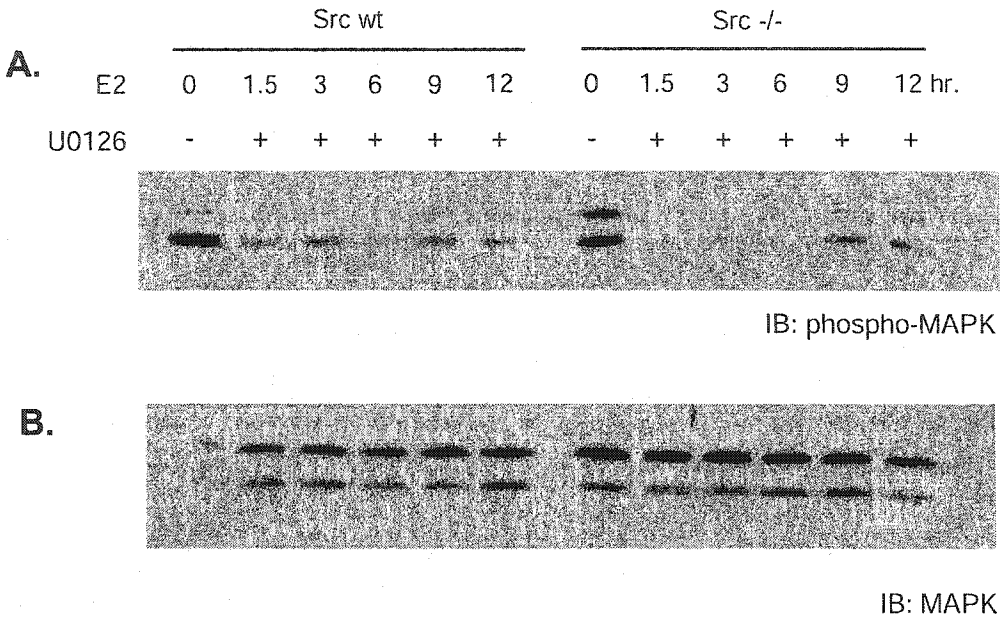
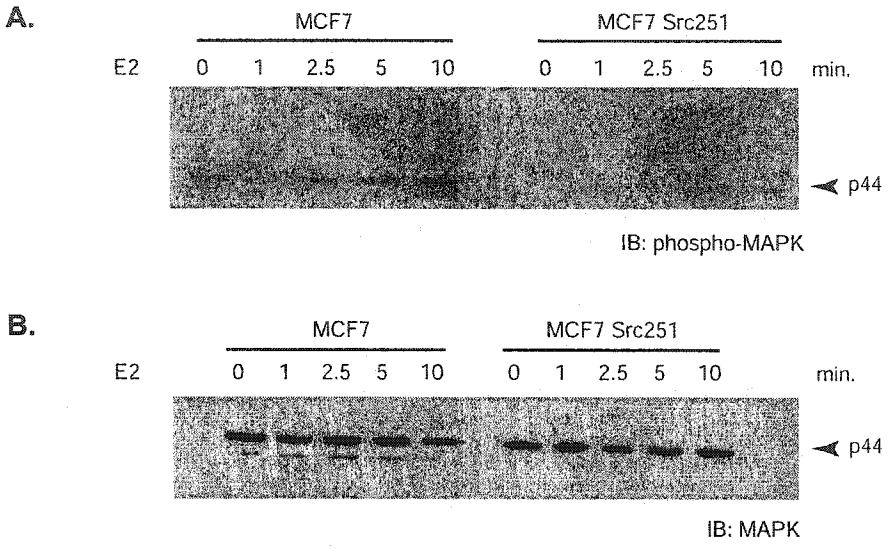

A.
Src wt
Src -/-
E2 0 1.5 3 6 9 12 0 1.5 3 6 9 12 hr.
U0126 - + + + + + - + + + + +
IB: phospho-MAPK
B.
IB: MAPK

Figure 4.12 Defect in MAPK activation upon E2 stimulation in the presence of dominant negative c-Src.

(A) MCF7 cells that express Src251 were stimulated with 100nM of E2 for various times. (B) Immunoblot analysis reveals that while MCF7 Src251 cells do not show an increase in MAPK activity, MCF7 parental cells show an increase at 10 hours post-stimulation. (C) The differences in activated MAPK levels is not due to differences in total MAPK.


A.
MCF7
MCF7 Src251
E2
0 1 2.5 5 10 0 1 2.5 5 10 min.
p44
IB: phospho-MAPK
B.
MCF7
MCF7 Src251
E2
0 1 2.5 5 10 0 1 2.5 5 10 min.
p44
IB: MAPK

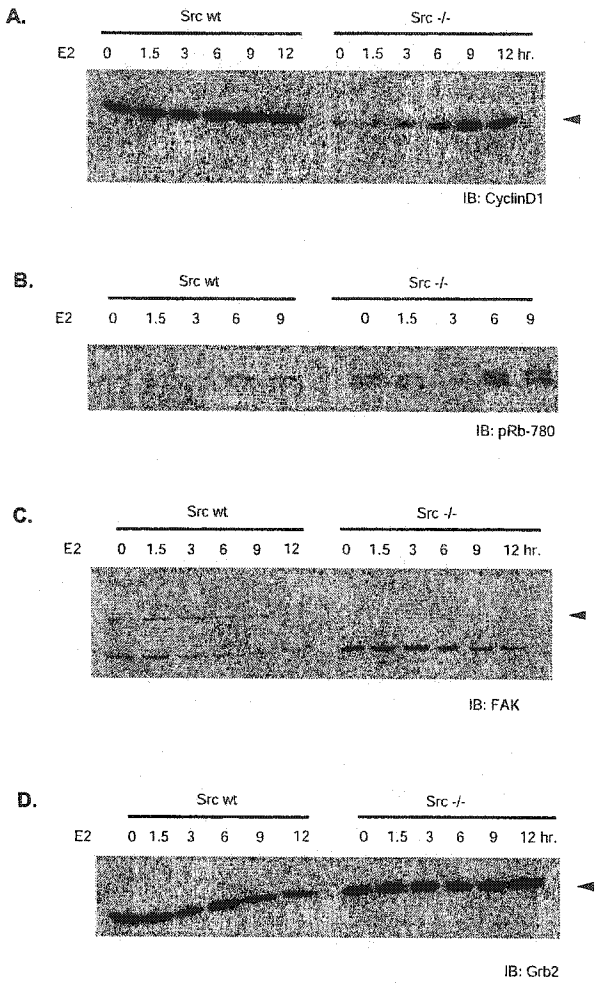
4.2.6 CyclinD1 levels are altered in c-src null mammary gland explants upon estrogen stimulation

Estrogen stimulation can promote the transition from G1 to S within the cell cycle, this event typically involving the induction of cyclinD1 and the activation of its associated kinases, cdk4/6. As one can induce cellular arrest with the use of antiestrogens, this effect can be rescued with the overexpression of cyclinD1, suggests that cyclinD1 plays an important role in estrogen mediated cell cycle progression (Prall et al., 1998; Prall et al., 1997; Sicinski et al., 1995). The importance of cyclinD1 in mammary gland development was suggested with the generation of a MMTV/cyclinD1 transgenic mouse. These mice are found to develop focal hyperplasias that develop into tumors with a long latency period. Consistent with its role in the mammary gland, cyclinD1 deficient mice displays a lactation defect. To explore whether cyclinD1 is affected in the c-*src* deficient MECs, the levels of cyclinD1 was measured following stimulation with estrogen (Figure 4.13a). Interestingly, the results of these studies reveal that baseline levels of cyclinD1 are lower in the *c-src* deficient epithelium compared to wild type epithelium. Furthermore, while the levels of cyclinD1 in the *c-src* deficient cells rose to wild type levels at 9 hours post stimulation, estrogen had little effect on the levels of cyclinD1 in the wild type cells. No significant difference in RNA levels of cyclinD1 was detected in c-*src* wt versus c-*src* null samples (Table 4.2). The differences in the levels of cyclinD1 were not due to differential protein recovery since both wild type and *c-src* deficient epithelium possess comparable levels of an adaptor protein, Grb2 (Figure 4.13d).

Cell culture experiments have suggested that an upregulation of the kinases associated with the cyclins occur upon stimulation with estrogen. Indeed, estrogen stimulation has been shown to result in a modest increase in cyclinD1 levels as well as an increase in cdk4/6 activity. The resulting increase in cdk activity correlates with the phosphorylation of the cdk target, the retinoblastoma (Rb) protein. Specific residues appear to be consistently phosphorylated and correlate with Rb function, one of these being serine 780. In order to address if there is an increase in cdk activity upon stimulation with estrogen in the MEC explants, cells were stimulated with estrogen and

Figure 4.13 Effects of a loss of c-*src* on cyclinD1, cdk4/6 activity, and FAK on MECs.

Mammary gland explants from wild type and c-*src* -/- mice were cultivated in CSS, serum starved and exposed to 100nM E2 over a defined timecourse. The levels of cyclinD1 are shown for each timepoint as well as the ability of cdks to phosphorylate a fragment of RB as a substrate, this being an indirect method to assess cdk associated activity with cyclinD1. The level of FAK is also shown at specific timepoints along with Grb2.


A.
Src wt
Src -/-
E2 0 1.5 3 6 9 12 0 1.5 3 6 9 12 hr.
IB: CyclinD1
B.
Src wt
Src -/-
E2 0 1.5 3 6 9 0 1.5 3 6 9
IB: pRb-780
C.
Src wt
Src -/-
E2 0 1.5 3 6 9 12 0 1.5 3 6 9 12 hr.
IB: FAK
D.
Src wt
Src -/-
E2 0 1.5 3 6 9 12 0 1.5 3 6 9 12 hr.
IB: Grb2

the phosphorylation status of Rb was investigated (Figure 4.13b). Upon estrogen stimulation a slight increase in Rb phosphorylation is observed, moreover it appears that a greater increase in Rb780 phosphorylation is observed with the loss of c-Src. This suggests that within this cell system the activity of cdks may be independent of c-Src and cyclinD1. Collectively, these observations argue that the loss of c-Src negatively impacts on the ability to induce cyclinD1 expression in an estrogen mediated time dependent manner, however its impact on the progression of the cell cycle as measured by the proteins associated with this event is unclear.

4.2.7 FAK levels are altered in c-src null mammary gland explants upon estrogen stimulation

One protein that has been implicated to play an important role in focal adhesion formation is focal adhesion kinase (FAK) (Lin et al., 1997; Schaller et al., 1994; Thomas et al., 1998). Significantly, the association of c-Src to FAK appears to be critical for the efficient propagation of a downstream Ras signal (Schlaepfer et al., 1997; Schlaepfer et al., 1994), suggesting that focal adhesions can mediate the activation of the Ras signaling pathway via c-Src. To investigate whether the loss of c-Src can modulate focal adhesions and possibly translate into differences in cell migration, FAK levels were measured from cells derived from mammary gland explants stimulated with estrogen (Figure 4.13c). Interestingly, while wild type cells display steady state levels of FAK, c-*src* null MECs display a two-fold decrease in FAK mRNA levels which correlate with a decrease in FAK protein levels (Figure 4.14b). This suggests that within MECs, c-Src appears to play a role in regulating FAK levels at the transcriptional level, further suggesting a general role for c-Src in an epithelial cells ability to associate with it and the substratum.

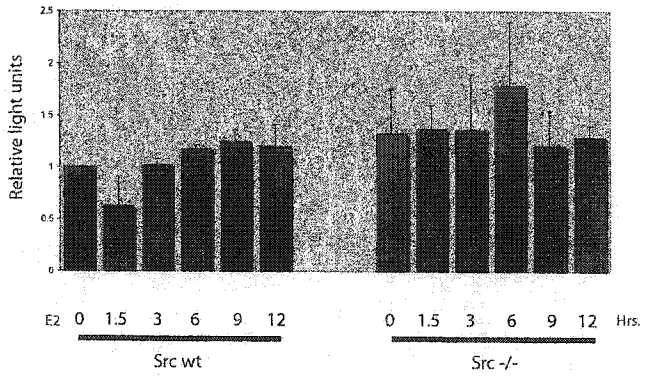
4.2.8 Changes in GSK3β phosphorylation observed in mammary glands from c-Src null mice

It is clear that the loss of c-Src affects the levels of ERα and cyclinD1 upon stimulation with estrogen, however the molecular mechanism remains to be elucidated.

Figure 4.14 RNA levels of cyclinD1 and FAK upon stimulation with estrogen in MECs.

Mammary gland explants from wild type and c-*src* -/- mice were cultivated in CSS, serum starved and exposed to 100nM E2 over a defined timecourse. RNA was isolated and (A) cyclinD1 RNA levels and (B) FAK RNA levels at each timepoint was assessed using oligonucleotides to amplify a specific product. Refer to Table 4.3 and 4.4 for details of each run. All data was then measured relative to wild type MECs at $time_0$.

A.


Relative light units
2.5
2
1.5
1
0.5
0
E2 0 1.5 3 6 9 12
Src wt
0 1.5 3 6 9 12 Hrs.
Src -/-

B.

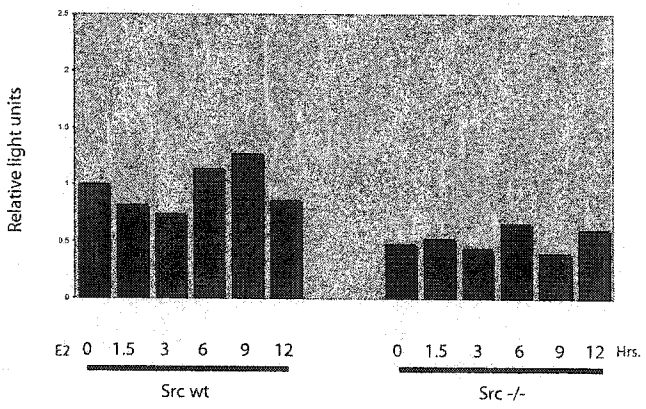

Relative light units
2.5
2
1.5
1
0.5
0
E2 0 1.5 3 6 9 12
Src wt
0 1.5 3 6 9 12 Hrs.
Src -/-

Table 4.2 CyclinD1 RNA levels

		Src wt						Src null						
	E2	0	1.5	3	6	9	12	0	1.5	3	6	9	12	Hrs.
CyclinD1-run 1 a		3.83	3.97	4.00	4.04	3.52	4.26	4.77	4.80	3.74	4.31	4.49	4.44	
CyclinD1-run 2 a		4.64	2.50	5.27	4.67	4.82	6.69	9.13	7.39	8.09	8.56	8.10	6.08	
PGK a		3.21	4.00	3.45	2.84	2.52	3.43	3.89	3.35	3.22	2.68	3.87	3.09	
CyclinD1/PGK-run 1 b		1.19	0.99	1.16	1.42	1.40	1.24	1.23	1.43	1.16	1.61	1.16	1.44	
Normalized to wt@0 c		1.00	0.83	0.98	1.20	1.17	1.04	1.03	1.20	0.98	1.35	0.97	1.21	
CyclinD1/PGK-run2 b		1.44	0.62	1.53	1.65	1.92	1.95	2.35	2.20	2.51	3.20	2.09	1.97	
Normalized to wt@0 c		1.00	0.43	1.06	1.14	1.33	1.35	1.63	1.53	1.74	2.22	1.45	1.37	
CyclinD1		1.00	0.63	1.02	1.17	1.25	1.20	1.33	1.37	1.36	1.79	1.21	1.29	
+/- stdev d		0.00	0.28	0.06	0.04	0.11	0.22	0.42	0.23	0.54	0.62	0.34	0.11	

a Numbers that represent the area under the specific peak for each amplification reaction.
Each area under the curve was read at least twice and the average calculated.

b Areas under the specific peak for each amplification reaction was normalized against the internal control PGK for each independent time point.

c All independent ratios for each timepoint was standardized relative to the ratio of wild type MECs at time 0.

d Average area under the specific peak for each amplification reaction normalized against PGK
and weighted to Src wt at time 0 +/- standard deviation.

Table 4.3 FAK RNA levels

		Src wt						Src null						
	E2	0	1.5	3	6	9	12	0	1.5	3	6	9	12	Hrs.
FAK [a]		2.89	2.96	2.30	2.91	2.88	2.66	1.67	1.60	1.29	1.60	1.39	1.68	
PGK [a]		3.21	4.00	3.45	2.84	2.52	3.43	3.89	3.35	3.22	2.68	3.87	3.09	
Ratio FAK/PGK [b]		0.899	0.738	0.667	1.026	1.144	0.775	0.430	0.476	0.399	0.596	0.358	0.544	
Normalized to wt@0 [c]		1.000	0.822	0.742	1.142	1.273	0.862	0.478	0.530	0.444	0.663	0.399	0.606	

a Numbers that represent the area under the specific peak for each amplification reaction.
Each area under the curve was read at least twice and the average calculated.

b Areas under the specific peak for each amplification reaction was normalized against the internal control PGK for each independent time point.

c All independent ratios for each timepoint was standardized relative to the ratio of wild type MECs at time 0.

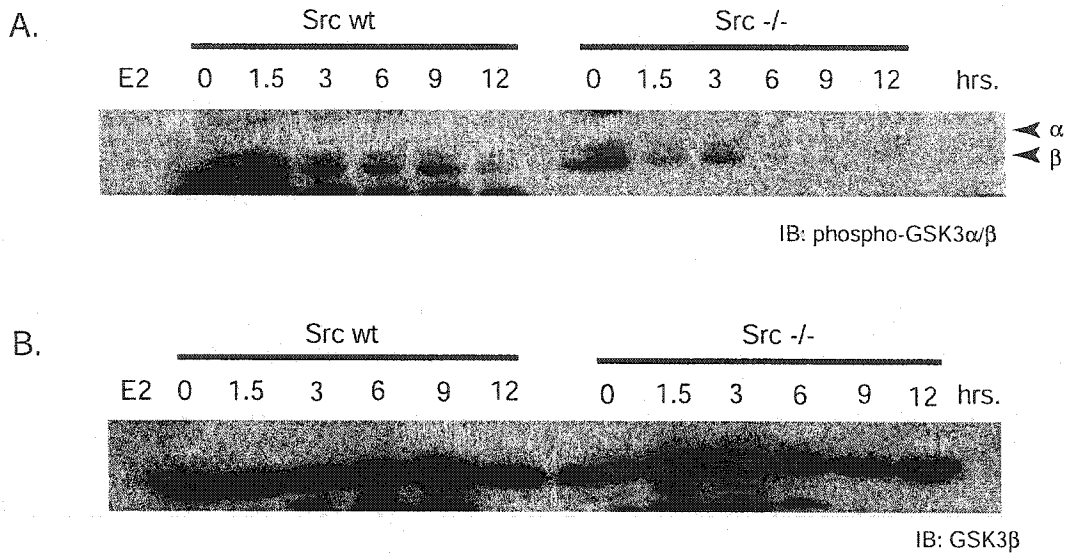
Estrogen receptor α and cyclinD1 levels are postulated to be controlled in a number of ways. For example, estrogen stimulation has been found to increase the transcription of cyclinD1 suggesting that a link between estrogen stimulation and transcriptional activation exists. Alternatively, recent evidence has suggested that protein stability may play a role in regulating cyclinD1 levels. Indeed, GSK3β has been found to initiate cyclinD1 ubiquitination thereby targeting it for degradation. The phosphorylation of two regulatory sites on GSK3β, serine 21/9, inactivates its activity thus leading to an increase in target protein levels. To address whether GSK3β activity correlates with ERα and cyclinD1 levels, lysates derived from mammary gland explants from wild type and c-Src null MECs were subjected to immunoblot analysis with a phosphospecific GSK3β antibody (Figure 4.15). Interestingly, c-Src null MECs display a lower level of GSK3β phosphorylation when compared to wild type MECs suggesting that an increase in protein degradation may be occurring mediated by GSK3β in correlation with the absence of c-Src.

4.2.9 Phosphorylation of STAT3 in c-Src null mammary glands

It appears that c-Src can phenotypically impinge on reproductive function via the estrogen receptor, however other proteins that are associated with c-Src may play a role either directly or indirectly in attributing to the phenotype. For example, the family of signal transducers and activators of transcription (STAT) factors have been implicated to play a role in reproductive development and importantly have been identified as a substrate for c-Src (Cao et al., 1996). These molecules act downstream of a number of signaling cascades, upon phosphorylation STAT monomers dimerize and translocate to the nucleus to associate with GAS/IRES elements in order to transactivate transcription. Indeed, the targeted genetic ablation of STAT3 results in a mammary gland phenotype that is characterized by a defect in lactation. In contrast, the overexpression of STAT3 in the mammary gland has been found to be tumorigenic. The regulation of STAT function can be mediated by a number of phosphorylation events found on the molecule.

Figure 4.15 Effects of a loss of c-*src* on GSK3β function in MECs.

Mammary gland explants from wild type and c-*src* -/- mice were cultivated in CSS, serum starved and exposed to 100nM E2 over a defined timecourse. (A) GSK3β activity was assessed by immunoblotting with a phospho-GSK3 antibody that detects both the α and β form of the protein. (B) Differences in GSK3 phosphorylation is not due to differences in total levels of GSK3β.


A.
Src wt
Src -/-
E2 0 1.5 3 6 9 12 0 1.5 3 6 9 12 hrs.
α
β
IB: phospho-GSK3α/β
B.
Src wt
Src -/-
E2 0 1.5 3 6 9 12 0 1.5 3 6 9 12 hrs.
IB: GSK3β

Specifically, the phosphorylation of tyrosine 704 and serine 727 has been associated with STAT dimerization and transcriptional transactivation respectively.

In order to investigate the significance of STAT3 in estrogen mediated mammary gland development via c-Src, MEC explants were stimulated with estrogen and the status of STAT phosphorylation was observed (Figure 4.16). Interestingly, no significant change was apparent in serine 727 phosphorylation, whereas tyrosine 704 appeared to display a slight delay in phosphorylation when stimulated with estrogen. These observations suggest that the ability of STAT3 to dimerize may be compromised however its ability to transactivate genes that contain response elements is not.

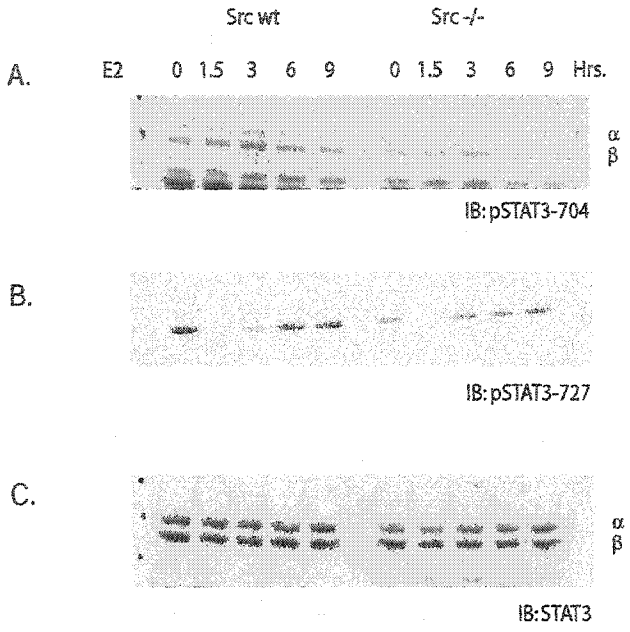
4.2.10 AKT phosphorylation in c-Src null mammary glands

It is clear that cell survival mediated by Akt is dependent on its translocation to the plasma membrane and subsequent activation via phosphorylation. One well-characterized pathway involved in Akt activation is the PI3'K pathway, the activation of PI3'K results in the modification of membrane lipids generating 3' phosphorylated phosphoinositides. These lipid modifications mediate the association between Akt and the plasma membrane via its pleckstrin homology (PH) domain (Bellacosa et al., 1998). The major enzymes involved in phosphorylating Akt at the membrane are the 3' phosphoinositide dependent protein kinases (PDKs). Activation of PDK-1 initiates the phosphorylation of Akt on Ser-308 and Ser-473. Both phosphorylation events appear to be necessary for the activation of Akt (Anderson et al., 1998). The activation of Akt results in the activation of downstream events associated with cell survival such as the phosphorylation of Bad, GSK3β, and the forkhead transcription factors (Brunet et al., 1999; Cross et al., 1995; Datta et al., 1997).

PI3'K activation has been speculated to be influenced by the association of Src family members to the p85:PI3'K subunit in a number of systems. For example, Lck can directly associate with the p85 subunit at tyrosine 668 (von Willebrand et al., 1998). However, these lines of evidence do not shed light on the specific role of c-Src in PI3'K activation. Recently the molecular consequence of p85:PI3'K tyrosine phosphorylation

Figure 4.16 Effects of a loss of c-*src* on STAT3 function in MECs.

Mammary gland explants from wild type and c-*src* -/- mice were cultivated in CSS, serum starved and exposed to 100nM E2 over a defined timecourse. (A) The ability of STAT3 to dimerize has been found to correlate with the phosphorylation of Y704. Phosphorylation of this residue was assessed by immunoblot analysis via a phospho-specific antibody that detects both the α and β versions of STAT3. (B) A similar analysis was done in order to assess the correlation between the loss of c-Src and the ability to transactivate via phosphorylation of S727. (C) Differences in phosphorylated proteins were compared to total levels of STAT3α or β.


Src wt
Src -/-
A.
E2 0 1.5 3 6 9 0 1.5 3 6 9 Hrs.
α
β
IB: pSTAT3-704
B.
IB: pSTAT3-727
C.
α
β
IB: STAT3

by c-Src has been elucidated. Data suggests that the p85:PI3'K subunit functions as a dual regulator with an intrinsic dominant inhibitory function, the phosphorylation of p85 by c-Src permitting its association of the p110 subunit thereby facilitating signal transduction (Chan et al., 2002).

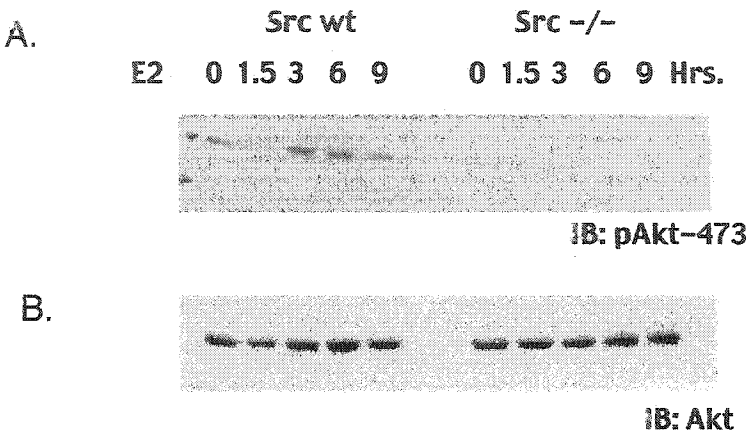
While the evidence points to a role for Akt in mammary gland development, the question of whether c-Src can influence Akt function and thereby modify the mammary gland architecture is unclear. To this end, mammary gland explants at various timepoints post-stimulation with estrogen was analyzed for the phosphorylation of serine 473, a residue characterized to correlate with Akt activity (Figure 4.17). In the absence of c-Src we found that Akt activity was suppressed at all time points relative to wildtype post-stimulation with estrogen. This suggests that the mammary gland defect may be influenced by machinery associated with apoptosis and cellular survival mediated by Akt.

4.3 Discussion

Although *in vitro* cell culture experiments suggest that formation of a c-Src/ERα complex is critical for estrogen action, the physiological importance of this interaction in the intact animal remains unclear. To further explore the *in vivo* significance between ERα and c-Src in normal mammary gland development, we performed a series of wholemount analyses of c-*src* deficient mice at various stages of mammary gland development. The results of these studies revealed that the female c-*src* null exhibited a dramatic defect in ductal outgrowth and terminal end bud formation that was further associated with a decrease in tyrosine phosphorylation of the ERα receptor. Furthermore, the primary mammary epithelium from c-*src* wild type mice displayed a rapid and sustained activation of the MAP in response to estrogen. In contrast, c-Src deficient mammary epithelium exhibited only a weak and transient response to MAP kinase stimulation. In addition, c-Src deficient MECs also displayed an impaired capacity to induce cyclinD1 and FAK expression. Consistent with these analyses, MCF7 cells expressing a dominant negative version of c-Src also were incapable of activating the MAP kinase pathway in response to stimulation of exogenous estrogens and displays

Figure 4.17 Loss of c-*src* attenuates Akt phosphorylation upon estrogen treatment of MECs.

Mammary gland explants from wild type and c-*src* -/- mice were cultivated in CSS, serum starved and exposed to 100nM E2 over a defined timecourse. (A) MECs were lysed and probed with anti-Akt S473, a phosphorylation site that correlates with the activation of Akt. (B) Differences in phosphoryated protein levels were compared relative to total levels of Akt.


A.
Src wt
Src -/-
E2 0 1.5 3 6 9 0 1.5 3 6 9 Hrs.
IB: pAkt-473
B.
IB: Akt

what appears to be a cell-substratum defect. Taken together these observations suggest that c-Src is critical for the mammary epithelium to respond to estrogens.

Given the fact that the loss of c-Src has a dramatic effect on normal mammary gland development, questions arise regarding the mechanism of how c-Src can create such a phenotype. Gross initial observations identify a significant decrease in the number of terminal end buds that correlate with the loss of c-Src. Within the terminal end bud lies two major cell populations, myoepithelial cells found at the ductal/stromal border and cap cells which are an undifferentiated cell type found just below the myoepithelial cell layer. As the terminal end buds migrate into the fat pad, cap cells differentiate and establish the ductal structures typical of a normal mammary tree. Given the decrease in terminal end bud numbers with the loss of c-Src, it is possible that the mammary gland defect is due to the inability to efficiently form ductile structures in comparison to wild type mammary glands. Interestingly, at 20 weeks of age the ductal architecture found in c-*src* null mammary glands generally appears indistinguishable to wild type suggesting that the phenotype may be representative of a delay rather than a defect. Nevertheless, it is unclear how the loss of c-Src affects the formation of terminal end bud structures in the developing mammary gland.

The results suggest strongly a general developmental delay, however the molecular mechanism that mediates this phenotype was unclear. The estrogen receptor was an obvious target for investigation due to its role in reproductive development in addition to its molecular association with c-Src. Upon further analysis of the estrogen receptor in MEC explants derived from c-Src null mice, it was discovered that the loss of c-Src negatively modulates estrogen receptor levels as well as its tyrosine phosphorylation. Significantly, the estrogen receptor gene contains an upstream ERE suggesting that it can modulate its own transcriptional activity. Given the importance of c-Src in estrogen receptor signaling, one can speculate that the absence of c-Src or the presence of the dominant negative may adversely effect the ability of the estrogen receptor to reach an optimal state of activation. This may manifest itself as an inability to efficiently phosphorylate the receptor resulting in a decrease in its activation.

Furthermore, the association of cofactors within the estrogen receptor complex may be negatively effected. If this is indeed the case, and given the fact that the ER modulates its own transcription via upstream EREs, a decrease in estrogen levels may be a consequence of a loss of c-Src modulating the estrogen receptor transcriptional complex.

The data suggests that c-Src is important in ERα signaling, however recent data utilizing deletion mutants suggest that tyrosine 537 may not be necessary for receptor dimerization and activation, suggesting a more general role for c-Src in ER signaling (White et al., 1997). For example, proteins found within the ER transcriptional complex such as p300/CBP (Kobayashi et al., 2000), steroid receptor coactivator (SRC-1) protein (Onate et al., 1995) and the receptor-interacting protein 140 (RIP-140) (Cavailles et al., 1994) differentially interact with the ER depending on its phosphorylation status (White et al., 1997). It is possible that the tyrosine phosphorylation of the ER at tyrosine 537 may result in the recruitment of specific coactivators, potentiating the ability of the ER to induce transcription.

4.3.1 *ER activation includes c-Src and Ras*

It is well established that c-Src impinges on the Ras pathway by a number of mechanisms. For example, c-Src has been shown to phosphorylate the adaptor protein Shc thereby providing a docking site for Grb2 and the activation of the Ras/MAPK pathway. Alternatively, c-Src can directly effect Raf activity (Stokoe and McCormick, 1997). Whether this phosphorylation event is crucial in the Ras/Raf interaction leading to activation is unclear, however it is speculated that the cooperative phosphorylation events of this tyrosine in addition to the well characterized serine/threonine phosphorylations on Raf may cooperate in the full activation of the MAPK pathway. The MAPK pathway also impinges on the ER via the phosphorylation of serine 118 found within the DNA binding region of the receptor. This phosphorylation event has been found to be sufficient to activate the receptor, leading to ERE binding and transactivation of downstream elements (Kato et al., 1995). Thus it is interesting that c-Src appears to play a key role in both activation events on the ER, firstly by phosphorylating the ER within the AF-2 region on

tyrosine 537 promoting dimerization, and secondly, by activating the MAPK pathway leading to the phosphorylation of serine 118 found within the AF-1 region. Both phosphorylation events appear to play an important role in the full activation potential of the receptor upon estrogen binding to the receptor. Recently, it has been reported that the presence of a dominant negative version of Src can negatively effect estrogen induced DNA synthesis (Castoria et al., 1999). Indeed, this does seem to be the case as MCF-7 cells that harbor Src251 display lower thymidine incorporation rates when exposed to estrogen (data not shown). We have now further shown that the absence of Src can negatively effect the development of the mammary gland. This points further to a general role for c-Src in playing a potential role in the activation of the ER, translating to a reproductive phenotype.

4.3.2 *Estrogen receptor activation and cell cycle proteins*

A recent report suggests a rather different role for cyclinD1 instead of its usual associated role with the cell cycle. Here, estrogen mediated activation of responsive genes was found to be independent of cdk activation. Furthermore, transcriptional activation of estrogen response genes can be mediated by cyclinD1 independent of estrogen. The presence of estrogen augments this transcriptional response. Finally, a physical association between the estrogen receptors HBD and cyclinD1 can increase the transcription of genes that are estrogen responsive (Zwijsen et al., 1997). Upon further analysis, steroid receptor coactivators (SRCs) were found to associate with cyclinD1 via a LxLL motif, potentially bridging the association between SRCs and the ER. In addition, this complex also contains the coactivator p300/CBP, the presence of both it and SRCs acting synergistically to activate transcription (Zwijsen et al., 1998).

Given the decrease in DNA synthesis observed by us as well as others (Castoria et al., 1999), one can also speculate that a slow down in the cell cycle may participate in a general lag in development. Indeed, of the cell cycle machinery the cyclins is one family that has been well characterized in regulating cell cycle progression. (DeFriend et al., 1994; Dubik and Shiu, 1988; Prall et al., 1998; Prall et al., 1997). Interestingly, MECs

derived from c-Src null mammary glands display a slight increase in the phosphorylation of the Retinoblastoma protein by cdks upon stimulation with estrogen. This appears counter to the hypothesis that the loss of c-Src may decrease the rate of transit through the cell cycle since an increase in Rb phosphorylation implies cell cycle acceleration. However, given the fact that stimulation with estrogen has multiple effects on proteins that play a role in cell cycle progression, it may be difficult to interpret how a loss of c-Src may impinge on estrogen mediated cell cycle progression. Indeed, estrogen stimulation has been found to not only alter cyclin expression, but also cyclin-dependent kinase inhibitors (CKIs) such as p21 and p27. Results suggest that while CKIs like p21 inhibit cyclinE/cdk2 function, p21 association with cyclinD1, depending on the stoichiometry between CKI and cyclinD1, can promote cell cycle progression (Pestell et al., 1999). Stimulation with estrogen thus can alter the expression, localization and the association of CKIs, G1 cyclins and their associated Cdks, thereby modulating cell cycle progression. The consequence of a loss of c-Src on cell cycle proteins and their role in regulating cell cycle progression is unclear however so far it appears to increase Rb phosphorylation. Proliferation assays using c-*src* null MECs as well as measuring the levels and activities of other cyclins and associated cdks respectively may shed more light on the role of c-Src on cyclinD1 and mammary gland development. CyclinD1 thus appears to play a dual role in the cell cycle, the first involving the initiation and progression of the cell cycle in the presence of estrogen. The second involving the estrogen mediated nucleation of a transcriptional unit comprising of cyclinD1, the estrogen receptor and its cofactors. This function not only has implications in development, but also in transformation and cancer, since many breast cancers have been found to overexpress cyclinD1. Furthermore, a recent report suggests that cyclinD1 is absolutely required for Neu mediated transformation *in vivo* (Yu et al., 2001). With its ability to couple to ER and its coactivators, one possibility is that cyclinD1 overexpression may promote resistance to antiestrogen therapies due to its ability to drive estrogen independent transcription.

4.3.3 *Estrogen, c-Src and GSK3β*

While transcriptional mechanisms may provide the most obvious means of regulating the levels of proteins associated with estrogen, an alternate mechanism may involve proteolysis. An interesting connection has been recently reported in that the Wnt pathway also cooperates with PgR signaling (Brisken et al., 2000). It has been well established that within the Wnt pathway, the molecule GSK3β can target proteins for degradation. An upstream protein, Desheveled negatively regulates GSK3β, thereby rescuing proteins that would otherwise be marked for degradation. Since PgR function has been found to require the direct cooperation of the ER (Migliaccio et al., 1998), the loss of Src will not only negatively affect the function of the ER, but also PgR function. Thus by uncoupling Wnt from GSK3β via a defective PgR/ER complex, proteins such as cyclinD1 may be targeted for degradation in Src deficient cells. Indeed, GSK3β has been found to phosphorylate cyclinD1 on Thr286, the result of which initiates the rapid turnover of cyclinD1 during S-phase via proteolysis (Diehl et al., 1998). The phosphorylation of Thr286 by GSK3β is also linked with the export of cyclinD1 to the cytoplasm in conjunction with the exportin protein CRM-1 (Alt et al., 2000). Similarly, proteosome inhibitors such as MG132 or lactacysin can block ER degradation suggesting that an ubiquitin-proteosome pathway can mediate the downregulation of the estrogen receptor (Nawaz et al., 1999). Furthermore, the formation of the ubiquitin complex has been found to also influence the stability of coactivators and corepressors bound to the estrogen receptor, suggesting a more broad role for proteosome mediated downregulation by estrogen (Lonard et al., 2000). While it is possible that the decrease in estrogen receptor levels found within the mammary gland of c-Src null mice may be mediated by protein degradation, it is unknown if this indeed is one of the mechanisms utilized. If this is the case, c-Src not only initiates the activation of the estrogen receptor, but also sets in motion its eventual proteolytic downregulation.

Alternatively, the activation of GSK3β by c-Src may occur via the PI3'K pathway. A substrate of c-Src, the p85 subunit of PI3'K, has recently been found to couple to the estrogen receptor pathway within the cytoplasm (Simoncini et al., 2000),

consequently leading to the activation of Akt (Campbell et al., 2001; Hisamoto et al., 2001). Indeed, the overexpression of Akt in the mammary epithelium results in a delay in regression (Hutchinson et al., 2001). Furthermore, stimulation with estrogen has been linked to the activation of Src, PI3'K, Akt and the Ras pathway in MCF-7 epithelial cells (Castoria et al., 2001). Therefore one can hypothesize that upon estrogen stimulation, c-Src can activate the estrogen receptor as well as multiple downstream pathways which includes the Ras/MAPK pathway and the PI3'K pathway. The latter pathway can via Akt block GSK3β and therefore stem the ubiquitinization of cyclinD1. This has indeed been found to occur since the downregulation of both PI3'K or Akt will lead to an increase in cyclinD1 stabilization (Diehl et al., 1998). While the evidence accumulates for the apparent crosstalk between the estrogen receptor, GSK3β, cyclinD1, Ras, Src, and PI3'K, whether or not these signaling events can all be mediated by estrogen is unclear. Interestingly, cyclinD1 levels do eventually return to normal levels upon estrogen stimulation in the absence of c-Src, suggesting that while Src is necessary for the stability of cyclinD1, it is definitely not sufficient. Given that previous reports suggest that cyclinD1 levels are regulated directly by GSK3β during the S-phase of the cell cycle, this implies that the role of GSK3β and cyclinD1 has less to do with early cell cycle events traditionally associated with cyclinD1, but more so with its potential transcriptional role as a scaffold protein with the estrogen receptor and its cofactors. Indeed, the loss of c-Src correlates with differences in Akt activation, a known regulator of GSK3β while not correlating with cdk4/6 activity.

4.3.4 *Function of Akt and STATs in mammary gland development*

Mammary gland development involves the massive remodeling of the fat pad stroma in the path of the invading ductal architecture with the onset of puberty. This process entails the rapid differentiation, proliferation and programmed death of cells within the mammary gland within a developmental window of approximately 6 weeks. With the establishment of the basic ductal mammary tree, the structure then falls into a quiescent state until pregnancy. The signaling pathways that are found to be involved in

the above processes that ultimately define the development of the mammary tree include such mediators as c-Src, the PI3'K pathway, Akt, GSK3β, the Ras/MAPK pathway and the cyclin/cdk's. While some of these proteins have been identified in proliferation, others such as Akt and the STATs have been implicated in apoptosis. Indeed, the activation of PI3'K initiates the activation of Akt via membrane association, in turn activating genes that play a role in cell survival. Similarly, biological and molecular evidence has suggested a role for the STAT family of transcription factors in mediating mammary gland development. STAT family members are expressed in a reciprocal fashion that correlates with the developmental stage of the mammary gland, with STAT5 potentially playing a survival role while STAT3 plays an apoptotic role in development and involution. Indeed, the generation and analysis of knockout mice for both STATs show that the loss of STAT5 results in an apoptotic phenotype while the loss of STAT3 results in a delay in involution due to a decrease in apoptosis.

Indirect evidence suggests that STAT3 function in the absence of c-Src displays a decrease in Y704 phosphorylation, suggesting that inefficient dimerization and DNA binding may translate into a decrease in apoptosis. However, there does not appear to be a change in transcription as assessed by the phosphorylation of serine 727. As a result, the defect in mammary gland outgrowth being due to an increase in apoptosis mediated by STAT3 does not appear to be a mechanism controlled by c-Src.

However, when detecting the level of Akt activation via phosphorylation of serine 473 we find that the loss of c-Src correlates with a decrease in Akt responsiveness when stimulated with estrogen. This suggests that c-Src may regulate survival pathways necessary during the developmental remodeling of the mammary gland. Recent data has indeed suggested that a complex exists between a ligand bound estrogen receptor, c-Src and the PI3'K. The association of this complex has been found to promote the S-phase entry of MCF-7 cells via cyclinD1/cdks upon stimulation with estrogen and is consistent with our *in vivo* data with the c-*src* null mammary gland further strengthening the existence of crosstalk between nuclear receptors and cytoplasmic factors.

4.3.5 *Stromal-epithelial interactions that define mammary gland formation during development*

Many published studies focus on the epithelial cell when studying estrogen receptor mediated signal transduction (Castoria et al., 1999; Migliaccio et al., 1996; Migliaccio et al., 1998; White et al., 1997), however it is becoming increasingly clear that the mesenchymal component of the mammary gland plays an equally important role in defining the development of the proper ductal structures during embryonic and postnatal development. Both epithelial cells and stromal cells express ER within the mammary gland (Haslam and Nummy, 1992), however reciprocal transplant experiments have demonstrated that estrogen receptor mediated ductal development is dependent on its presence in the stroma (Cunha et al., 1997). This suggests that a more complex interplay may exist between the mammary gland stroma and epithelium that defines the development of the mammary gland.

Our data demonstrates that the loss of c-Src in the mouse results in multiple physiological defects that impact on reproductive structures that include the mammary gland, the ovaries and the uterus. For example, ERα expression at the RNA and protein levels appear to decrease, the responsiveness of MAPK is delayed upon estrogen stimulation, and a lag in GSK3β, STAT3 and Akt activation is observed in MECs derived from c-*src* null mice as well as MCF-7 cells that express dominant negative c-Src. The MEC and the MCF-7 data suggest that the c-*src* mammary gland defect is epithelial. However, we also observe that reciprocal transplants point to a stromal component. The expression of c-Src has been documented to be ubiquitous (Soriano et al., 1991; Thomas and Brugge, 1997), and taken together these observations are reminiscent of the ERα transplant data.

Superficially, the cell culture data and the transplant data appear to be incongruent, however this is not surprising. By definition, estrogen receptor studies in cell culture with pure populations of epithelial cells would never shed light on the interactions that occur between epithelium and stroma that leads to normal mammary gland development. Nevertheless, what is the mechanism within the stromal component

that can compensate for the loss of c-Src and in turn a loss of ERα function in the epithelium? These factors may include the relative expression of ERα itself in each cellular compartment, the expression of other family members such as ERβ, differential expression of transcriptional cofactors in each cellular compartment or the presence of different upstream EREs that may define estrogen receptor function whether it is in the stroma or the epithelium.

Given these possibilities c-Src, and along the same line ERα, may either play a minimal role in the epithelium or a role that is not directly involved in ductal development. Evidence has shown that estrogen stimulation can induce EGF and EGFR expression in mammary tissues. Furthermore, reciprocal transplants utilizing EGFR null tissues demonstrate that EGFR function is absolutely necessary in the stroma to induce estrogen dependent ductal outgrowth (Wiesen et al., 1999). In light of the fact that c-ErbB2 can heterodimerize with the EGFR this places c-Src, ERα and EGFR/c-ErbB2 all within the stromal compartment of the mammary gland (Sabastian et al., 1998). Estrogenic conditions therefore appear to work in a paracrine fashion between the mesenchyme and the epithelium, estrogen activation being influenced by c-Src and MAPKs within the stroma, resulting in transactivation of ERE containing genes, notably EGF, that can influence epithelial cell growth and development.

The loss of c-Src within epithelial cells result in defects in ERα signaling and estrogen mediated downstream effects, however given that transplant data suggests that the loss of c-Src plays a minimal role in ductal development the effects that we see within epithelial cells may be compensated by other factors. One relatively new molecule that has been identified in estrogen receptor signaling is the estrogen receptor related (ERR) receptor (Giguere et al., 1988). This orphan receptor family displays moderate homology to the estrogen receptor and has been shown to interact with EREs. Expression studies have identified ERRα in tissues such as bone, brain and central nervous system, heart and muscle. Breast cancer cell lines have also been shown to express ERR receptors however expression within the mammary gland is unclear let alone whether or not it is expressed in either the stroma or the epithelium. If ERR receptors are expressed within epithelial

cells and not the stroma then this may compensate epithelial function. Indeed, a similar hypothesis has been suggested with regards to bone in that the relatively low levels of ERα are compensated by high levels of ERR receptors thus accounting for the sensitivity of bone to estrogen, or the lack thereof (Vanacker et al., 1999).

The other possibility is that the presence or absence of factors independent of estrogen receptor signaling may influence the architecture of the mammary gland. For example, the naturally occurring null mutant mouse $Csfm^{op}$ is null for the ligand CSF1, a factor critical in tissue macrophage production, survival and growth. $Csfm^{op}/Csfm^{op}$ mice have a decrease in macrophage numbers that correlate with incomplete ductal outgrowth during lactation. Irradiation of mammary glands displays a phenotype similar to the $Csfm^{op}/Csfm^{op}$ mouse suggesting a role for macrophage function and more importantly reinforcing the role of the mesenchyme in normal ductal development. It is hypothesized that macrophages within the stroma generate growth factors that can influence tissue remodeling of the extracellular matrix within the mesenchymal space (Gouon-Evans et al., 2000). Interestingly, biochemical data with the CSF1 receptor show that receptor activation requires the association of c-Src (Alonso et al., 1995) further suggesting that c-Src plays an important role in ductal growth when in conjunction with other receptor systems.

4.3.6 *Chapter summary*

In this chapter, I have described a biological phenotype that suggests a role for the c-Src protein tyrosine kinase in normal mammary gland development. To examine the possibility that c-Src may play a role in normal reproductive development I investigated whether any reproductive abnormalities existed within the genetically ablated c-*src* mouse. Significantly, I discovered that c-*src* null virgin female mice display a mammary gland defect that includes a decrease in terminal end bud numbers in conjunction with a delay in ductal elongation. Analysis of uteri excised from wild type and c-*src* null mice at 3 and 6 weeks of age show that they are grossly dissimilar, the loss of c-Src correlating with a hypoplastic phenotype. Although all uterine cell types are present in c- *src* null

females, they display a reduction in their stromal, myometrial and epithelial tissue compartments. Furthermore, histological analysis of wild type ovaries from mice at 3 and 6 weeks of age display oocytes at all stages of development from primary to mature late stage Graafian follicles. In contrast, c-*src* null ovaries display only primary and secondary oocytes that do not display any late stage follicular development.

These observations suggest that c-Src may play a role in the development of reproductive tissues, however the molecular mechanism as to how this occurs is unclear. To address this, I analyzed mammary epithelial cell explants from wild type and c-*src* null mice. To this end, I observed that explants derived from c-*src* null mice display a decrease in the levels of ERα that correlates with a decrease in its tyrosine phosphorylation. These observations are supported by data that utilized a dominant negative version of c-Src in MCF7 cells, suggesting that c-Src has the ability to regulate ERα levels. This regulation appears to be at both the transcriptional and post-translational level. Further analysis reveals a defect in MAPK activation when mediated by estradiol from c-*src* null mammary gland explants as well as MCF-7 cells that harbor the dominant negative c-Src, suggesting that c-Src is critical in mediating the activation of both the estrogen receptor as well as the MAPK pathways. Finally, cell cycle and cytoskeletal proteins may play a role in the generation of the reproductive phenotype. Specifically, cyclinD1 levels appear to be suppressed in the absence of c-Src upon estrogen stimulation. The decrease in protein levels do not correlate with a decrease in mRNA levels, however they do correlate with a delay in GSK3β inactivation, suggesting that the delay in cyclinD1 expression in the absence of c-Src may involve a defect in cyclinD1 stability or turnover. In addition, the loss of c-Src correlates with a decrease in FAK levels, while the presence of a dominant negative version of c-Src correlates with a cell-substratum defect suggesting that the cytoskeletal architecture may be negatively influenced by a loss of c-Src. Taken together, I have characterized a novel phenotype within the c-*src* null mouse that provides a biologically significant connection between c-Src function and estrogen receptor action within reproductive tissues

The knockout mouse has brought clarification to the function of many genes, however the technique still has weaknesses, the primary problem being the global deletion of the gene in question. Given the massive complexity of normal physiological processes, it is very possible that the deletion of a gene in one tissue will negatively impact the function of another tissue. This is actually exemplified by the ERKOα mouse, where the loss of the receptor generates multiple reproductive phenotypes, however, many of these phenotypes are due to defects in the hypophesial-pituitary axis, stressing the relationship of neuronal signaling and systemic factors and their influence on target tissues. In order of circumvent this problem, a number of techniques have been developed whereby genes can be altered or expressed in a specific tissue. One way to address the tissue specific role of c-Src in the mammary epithelium is to therefore generate a dominant negative c-Src transgenic mouse driven by the MMTV promoter. While this strategy would be useful in addressing the role of c-Src in the mammary epithelium, a number of problems arise with the use of this technique in development. One complication is the sensitivity of the MMTV promoter to hormonal factors indeed present in the mammary gland. The interpretation of the outcome may be unclear due to this fact. Another possibility may rely on the homologous recombination of a construct that is under the control of an inducible excision system, whereby the presence of the excision mechanism driven by tissue specific promoters induce a loss-of-function in that tissue of interest. To further investigate the role of c-Src in the mammary epithelium, a floxed c-Src construct may address the necessity of this tyrosine kinase in mammary gland development. Similarly, ERα function can be addressed utilizing a floxed ERα excised specifically in the mammary gland.

Intra-tissue communication between distinct cell types to properly organize the mammary gland is an important aspect to acknowledge. Previous data have pointed to the presence of the estrogen receptor in both the stromal and epithelial components of the mammary gland. As to which compartment is important in the development and maintenance of the mammary gland is unclear, however it is very possible that signaling from the estrogen receptor is important in both compartments. Alternatively, estrogen

receptor function may only be required in an individual tissue compartment. To distinguish between these two possibilities, tissue recombinants were generated utilizing combinations of ERKOα and wild-type tissues from each mammary gland compartment. To this end, it was found that estrogen mediates its effects from the stroma, the estrogen receptor in the epithelium while present and functional was not necessary (Cunha et al., 1997), thus relying on cell-cell communication to influence the epithelial structures of the gland. Given this result, it is unclear if the mammary gland phenotype due to the loss of c-Src function is a result of its absence in the mammary gland stroma or the epithelium. The phenotype of the c-Src null mammary gland suggests that the inability to support the normal growth of the epithelial structures are due to the inefficient functioning of the estrogen receptor via c-Src. A number of experiments can help resolve this issue. For example, the specific expression of a dominant negative c-Src, driven by the epithelial specific MMTV promoter can be derived. Alternatively, tissue recombination experiments within the mammary gland provides a more elegant technique to address the question. Results from this experiment suggest that a stromal component can mediate the difference in ductal outgrowth in c-*src* null mammary glands. Indeed, there is a clear difference in growth potential at 3 weeks when comparing the ductal outgrowth of c-*src* null mammary glands and transplanted c-*src* null glands at the equivalent time point relative to wild type controls. The above data is consistent with the role of the estrogen receptor in the stroma and places c-Src within the same compartment to functionally influence ductal outgrowth. This further implies that molecularly, the defect in the c-*src* null mammary gland may be due to the inability to efficiently activate stromally derived ERα.

Numerous lines of evidence show that ERα plays a significant role in mediating growth and transcriptional activation in epithelial cells. However all the events that occur appear to not be necessary nor sufficient in mediating ductal outgrowth. The EGFR and c-Src also appear to be non-autonomous in its function in mammary gland development implicating the mesenchymal component in directing epithelial architecture via paracrine communication. In addition, factors independent of estrogen receptor or mesenchyme

such as macrophages have been found to modify and remodel the extracellular matrix during ductal growth. The epithelial component while being fully responsive to estrogen appears to play a passive role in ductal formation, taking its developmental cues from the stroma. What is the functional role of ERα in the epithelium? Estrogen stimulation of epithelial cells may play a role in the genomic and non-genomic activation of genes involved in cell survival and mitogenesis, with the coordination of ligand stimulated epithelial cell proliferation and stromally derived signals dictating the ductal architecture. Indeed, expression of Akt in the mammary gland confers epithelial cell survival, decreased apoptosis and a delay in mammary gland involution (Hutchinson et al., 2001). Similarly, estrogen stimulation upregulates PI3'K, Akt and c-Src activity while in contrast, inhibition of c-Src results in the prevention of estrogen stimulated PI3'K and Akt activation (Castoria et al., 2001).

CHAPTER 5

Summary and future directions

5.1 Importance of c-Src in RTK signaling

The ability to activate receptor tyrosine kinases has been found to involve the association of specific ligands to the binding region of the receptor, augmenting the aggregation of receptor monomers into active signaling complexes. The proximity of ligand bound receptors to each other initiates the conformational change required to drive intracellular signaling. Currently, the general model involves not only changes in receptor structure thereby facilitating the acceptance of ATP within the kinase region but in addition the modification of specific residues that are found in and around landmarks such as the activation loop. The phosphorylation of these structures have been found to play a critical role in facilitating the full activation of the kinase, potentially by the modification of the activation loop into a more stable conformation, prolonging receptor activation. This has indeed been found to be the case as demonstrated by the FGFR and IR systems (Ellis et al., 1986; Mohammadi et al., 1996). In these examples, we find that receptor activation involves the association of ligand to the receptor thereby increasing the local concentration and proximity of the receptor cluster. The activation of the kinase region has been found to involve the stabilization of the flexible activation loop, which appears to be in a conformational flux of either open or closed, potentially increasing the possibility of the introduction of ATP to the binding pocket (Hubbard et al., 1998). The association of ATP to the pocket activates the receptor and induces the reciprocal trans-autophosphorylation of the opposite receptor at specific residues thereby stabilizing the activation loop.

While this order of operation may indeed explain the reciprocal auto-phosphorylation of receptor clusters, other mechanisms may be involved such as the direct association of cytoplasmic proteins facilitating the phosphorylation and

stabilization of the activation loop. This may indeed be the case since phosphatase inhibitors induce the constitutive activation of receptor complexes independent of ligand (Posner et al., 1994) implying that phosphatases, and potentially kinases can regulate receptor activation. The evidence I have presented involves the association of the c-Src protein kinase to the c-ErbB2/Neu receptor. The current literature so far suggests that dimerization is absolutely critical for the function of this receptor family (Burke and Stern, 1998). However it is unclear if the same mechanism of activation i.e. activation loop phosphorylation, is utilized in the EGFR family as is found in either the FGFR (Mohammadi et al., 1996) or IR (Ellis et al., 1986) system. Biochemical evidence derived from human and mouse tumor models suggest an importance for the presence of c-Src in transformation. c-Src is overexpressed in MMTV derived tumors overexpressing activated versions of Neu (Muthuswamy et al., 1994), and may account for at least 70% of the kinase activity found in human epithelial cancers (Ottenhoff-Kalff et al., 1992). These observations also correlate with the direct association of c-Src to the receptor. Given these observations, the significance of the association of c-Src to this receptor family is unclear. The surprising find is the fact that c-Src appears not to associate with the regulatory region of the receptor but with the activation loop found within the receptor. Conversion of this tyrosine ablates Neu mediated transformation (Zhang et al., 1998), whereas the same tyrosine found in the EGFR does not appear to influence receptor function (Gotoh et al., 1992).

The evolution of receptor systems appears to have included mechanisms with which to inhibit spurious activation of kinase activity, independent of ligand. The example set by the FGFR and IR families appear to not be unique in that EGFR family members follow the same mechanism of autoinhibition. The most drastic example may be provided by c-ErbB3. Indeed, this receptor member appears to have eliminated kinase activity altogether in spite of the fact that it possesses a specific ligand. The restoration of activity with the generation of an EGFR that possesses the carboxyl terminal region of c-ErbB3 displays an extremely potent transforming capacity, suggesting that c-ErbB3 may have played a critical role in signaling and potentially transformation (Waterman et al.,

1999). Tumors derived from transgenic mice that overexpress an activated version of Neu also display extremely high levels of c-ErbB3 (Siegel et al., 1999). One can speculate that over the course of evolution the potency of c-ErbB3 may have been downregulated by kinase inactivation. Likewise, the c-ErbB2 receptor displays a high degree of activity demonstrated both in cell culture and *in vivo* within the mammary gland translating into tumorigenesis. With the preference for c-ErbB2 as a dimerization partner (Graus-Porta et al., 1997) in conjunction with its high catalytic activity (Di Fiore et al., 1987b), mechanisms may have evolved to tightly regulate this receptors activity. As drastic as c-ErbB3 in lacking catalytic activity, c-ErbB2 activity may have been attenuated with the absence or elimination of a specific ligand. Furthermore, the regulation of the activation loop may have been placed under a similar mode of regulation as found in the IR and FGF receptor systems, in the case of c-ErbB2, activation loop phosphorylation being controlled by c-Src. Interestingly, the homologous tyrosine found in the activation loop of the EGFR does not appear to play as important a role in regulating receptor activity (Gotoh et al., 1992; Hubbard et al., 1998). In this case, given the fact that the EGFR possesses a specific ligand, in addition to its lower kinase activity as assessed by transformation assays (Di Fiore et al., 1987a; Di Fiore et al., 1987b), further regulation of the receptor may not be required.

To further the hypothesis that the tyrosine found within the activation loop of the c-ErbB2/Neu RTK plays an important role in human disease, it will be of interest to screen for mutations in diseases that have been associated with the overexpression and/or amplification of the receptor. Indeed, other receptor systems have been found to harbor various mutations within the region of the activation loop, such as the Met receptor (D1246N, Y1248C) or the FGFR3 (K650E) resulting in patients with hereditary predisposition to develop multiple papillary renal-cell carcinomas (HPRCC) (Miller et al., 2001) or thanatophoric dysplasia type II (TDII) (Naski et al., 1996) respectively, both resulting in higher receptor kinase activity. These mutations have been hypothesized to activate the receptor by generating a conformation that mimics the activated loop rather

than mimicking the phosphorylation required for activation, nevertheless it appears that this region of the receptor is prone to somatic mutations associated with human diseases.

5.2 ERα and c-ErbB2 in mammary tumorigenesis

The importance of c-Src in c-ErbB2 signaling is inferred both in binding studies as well as dominant negative assays. These observations directly impact on the significance of these two proteins in the establishment and progression of epithelial cell transformation and tumorigenesis. The role of c-ErbB2/Neu is well established as an instigator of transformation, and in conjunction with c-Src provides a potential mechanism of receptor regulation. Interestingly, mammary gland transformation while being extremely sensitive to the presence of c-ErbB2 is also under tight regulation by the estrogen receptor. Evidence suggests an inverse correlation in that high c-ErbB2 levels are linked to the acquisition of an anti-estrogen resistant phenotype and a poor patient prognosis (Newby et al., 1997). Associated with both the estrogen receptor and c-ErbB2 is the c-Src kinase which appears to play important roles in the function of both receptors. Does the crosstalk between these two receptors translate into a phenotype?

One study that has addressed the importance of the estrogen receptor in mammary gland tumorigenesis involved the transforming potential of the Wnt pathway (Kwan et al., 1992). With the recent identification of the Wnt pathway in hormone signaling, transgenic mice that overexpress Wnt-1 in the mammary gland via the MMTV promotor was interbred with ERKOα mice to address the role of the estrogen receptor in Wnt mediated tumorigenesis (Bocchinfuso et al., 1999). Interestingly, both MMTV-Wnt1 transgenic mice that either express the estrogen receptor or are ERα null developed hyperplasias, suggesting that the estrogen receptor is not required for the hyperplastic growth of mammary tissues. However, the time of onset was significantly different in that MMTV-Wnt1:ERKOα transgenic mice developed hyperplasias at a delayed rate than MMTV-Wnt1 controls (Bocchinfuso et al., 1999). Similar results were observed with MMTV-c-ErbB2:ERKOα transgenic mice (Hewitt et al., 2002). While these observations are significant, a simple and alternative explanation to this result should take into account the inherent mammary gland defect found in the ERKOα mice. Nevertheless, these

observations have a number of implications on the role and importance of the estrogen receptor on mammary gland transformation. The observation from mouse models suggesting that the absence of the estrogen receptor in the mammary gland significantly delays tumor formation is in agreement with the clinical rationale for the use of antiestrogens as an adjuvant in the treatment of breast cancer. However, both MMTV-Wnt1:ERKOα and MMTV-c-ErbB2:ERKOα transgenic mice still develop hyperplasias suggesting that while the estrogen receptor may play a significant role in the regulation of cell growth and development, it is not sufficient to significantly modulate or in this case attenuate tumor growth. Furthermore, this suggests that while the estrogen receptor as a target for therapy is clinically efficacious, it is in the long term only a temporary avenue for cancer control. In spite of the above animal model it is well known and has been reiterated many times that breast cancer samples that are ERα negative are more aggressive, apparently in contrast to the MMTV-Wnt1:ERKOα and MMTV-c-ErbB2:ERKOα transgenic model which shows that tumor development is delayed. However, the definition of 'aggressive' may be contextual in that these tumors are basically uncontrollable by current regimes and similar to the animal model, the development of hyperplasias and potentially overt carcinomas is inevitable. It will be of interest to see whether other oncogenes implicated in mammary gland transformation can potentiate tumorigenesis in the absence of ERα. In line with the current data presented in this thesis it would be of interest to test the status of the c-Src PTK in these tumors. An alternate experiment would be to generate MMTV-Neu:c-*src* null mice and investigate the status of ERα.

The development of cancer involves the stepwise accumulation of genetic events that ultimately leads to the deregulation of normal cellular function. Both the ERα and c-ErbB2 play important roles in breast cancer progression and both have been found to be inversely related, in that estrogen can downregulate c-ErbB2 expression (Dati et al., 1990) and conversely, high levels of c-ErbB2 can negatively impact ERα expression (Newman et al., 2000). In addition, both receptor systems now appear to share common cytoplasmic signaling components. Indeed, even though both c-ErbB2 and ERα inversely

regulate each other the activation of either receptor can lead to the same outcome, signal transduction and gene transcription. One could thus envision that early events in transformation include the co-overexpression of ERα and c-ErbB2 followed by a progressive destabilization of the cell. The accelerated mitotic index has been found to result in an increase in mutational frequency due to inefficient DNA replication/repair mechanisms (Liu et al., 2002). This may induce c-ErbB2/ERα overexpression thereby exacerbating the transformed phenotype. In this case these tumors would be c-ErbB2 and ERα positive and amenable to antiestrogen therapy. However, given that c-ErbB2 can competitively inhibit ERα expression it is possible that high levels of c-ErbB2 will decrease ERα levels to a point where a tumor may be described as ERα negative. Here, we can predict a decrease in the response to antiestrogens and at these advanced stages it may be considered antiestrogen resistant. Given the fact that both receptors can signal common pathways, it is reasonable to speculate that there would be no need for the expression of one receptor to reach levels high enough to suppress the other. Without any selective pressure, this situation may generate a transformed cell that is while extremely aggressive, controllable with TAM. Indeed, the efficacious nature of TAM results from the inhibition of transcriptionally activating functions of the ER via multiple signaling mechanisms. The possibility of a change in expression patterns may occur when selection pressure is applied, ironically in the form of antiestrogens. Here we may see that an even greater increase in the expression of genes such as c-ErbB2 with extended TAM treatment may consequentially downregulate ER, thus making TAM useless. Therefore, one may observe a gradual shift in ER levels when under this mode of selection. However, this shift in expression is not readily observed, suggesting that alternate mechanisms may confer TAM resistance.

5.3 Importance of c-Src in ERα and c-ErbB2: non-genomic signaling, MAPK feedback and stromal-epithelial interactions.

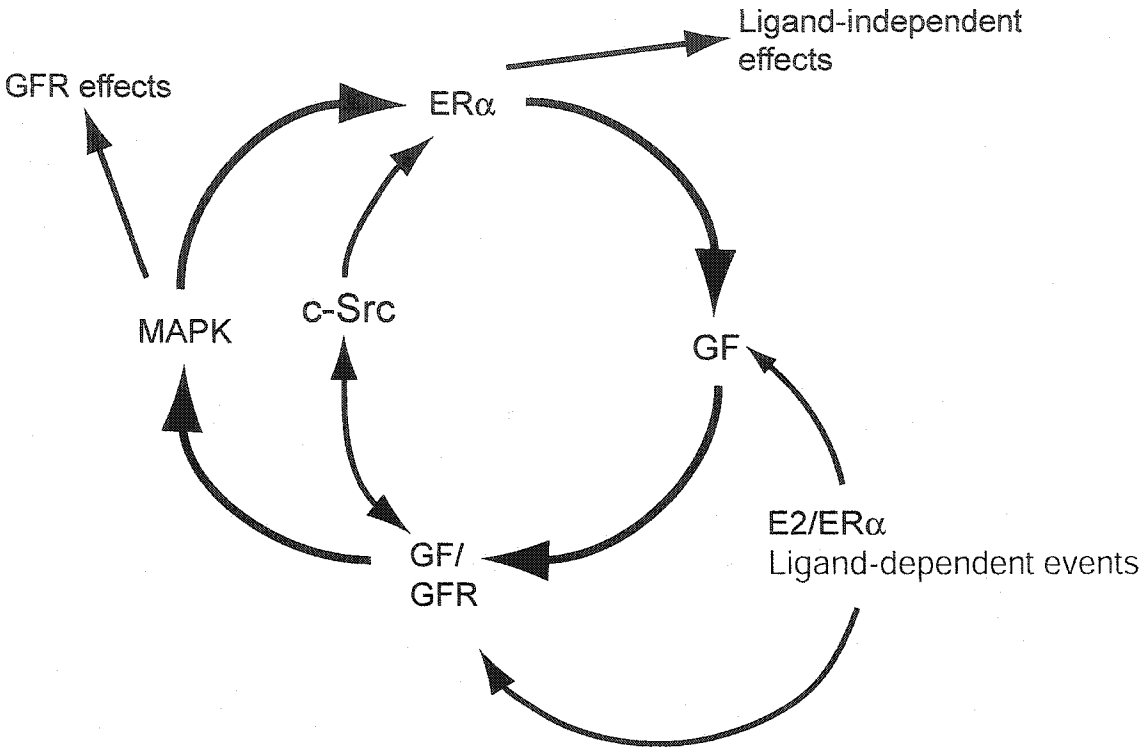
It is clear that mutational events leading to the deregulation of normal cellular function can lead to a pathological state. Signaling cascades such as those leading to the

Ras pathway and the Src kinases have been shown definitively to play a role in c-ErbB2 mediated transformation. The overexpression of c-ErbB2 has been found to inversely correlate with estrogen receptor levels, further accelerating the development of an aggressive tumor phenotype. Given the role of c-Src in estrogen receptor regulation, the increase of both c-ErbB2 and estrogen receptor levels in human breast cancer may also increase the significance of the role of c-Src in its ability to modify and modulate both these receptor functions. Indeed, the phosphorylation of the estrogen receptor may provide the means for the association of coactivators or corepressors to the transcriptional machinery thereby facilitating its regulation. The role of c-Src in the insuing crosstalk implies that a significant portion of the estrogen receptor resides in the cytoplasm. While classically associated with the nucleus, evidence now is accumulating that point to a significant role for the estrogen receptor in the cytoplasmic compartment. For example, estrogen stimulation is associated with the coimmunoprecipitation of both PI3'K and the estrogen receptor, this association leading to the activation of the Akt pathway (Castoria et al., 2001). Estrogen stimulation has also been linked to the activation of the MAPK pathway (Kato et al., 1995), however this may be indirect via growth factor receptor association with adapter proteins such as Shc and kinases such as c-Src (Migliaccio et al., 1996).

The significance of these associations within the cytoplasm are unclear, however it may play a role in the establishment of what is now referred to as non-genomic estrogen mediated signaling events. In contrast to the classic genomic events associated with the estrogen receptor these non-genomic events occur within minutes following estrogen stimulation and can involve the association of the ER to cytoplasmic signaling proteins as well as crosstalk between ERα and cell surface receptors (Figure 5.1). It appears then, that the estrogen receptor can play a casual role in short term signaling leading to immediate responses, as well as one that provokes long term genomic effects leading to transcription and cell cycle progression. These non-genomic events may initiate indirectly from estrogen-mediated expression of growth factor receptors (GFRs) or their ligands. Alternatively, receptor activation of GFRs may occur directly via for

Figure 5.1 Potential effects mediated by growth factor receptors, the estrogen receptor and cytoplasmic kinases.

Signaling pathways that have now been realized to crosstalk has been suggested to potentially result in cyclic events that may lead to the ligand-independent activation of the estrogen receptor. Key molecules that play roles in mediating this crosstalk include the c-Src PTK and MAPK. GF, growth factor; GFR, growth factor receptor.

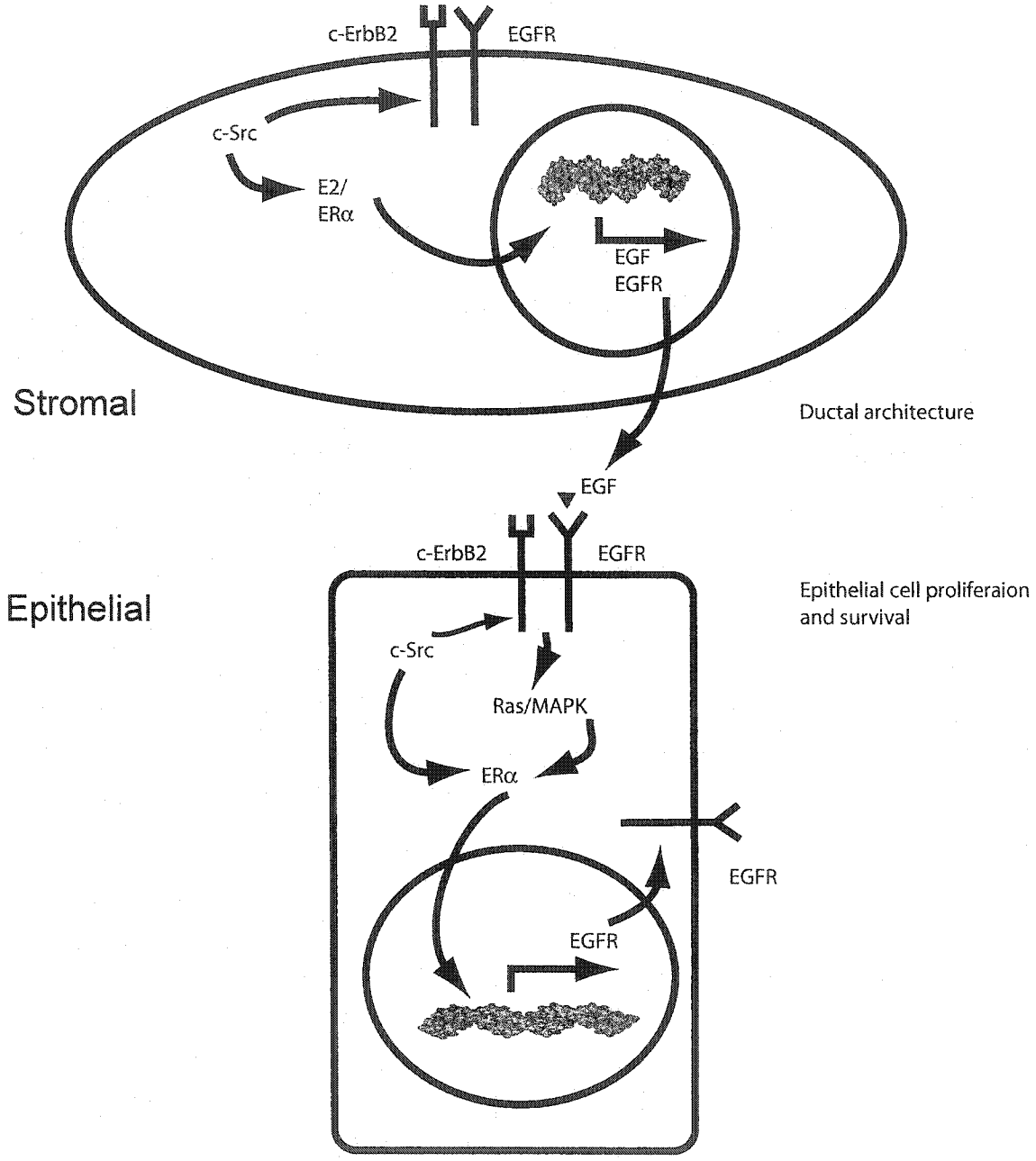

Ligand-independent
effects
GFR effects
ERα
MAPK
c-Src
GF
GF/
GFR
E2/ERα
Ligand-dependent events

example EGF. Initiation of GFR signaling activates the MAPK and c-Src pathways, both of which can modulate the activity of the ER. Activation of MAPK or c-Src results in the phosphorylation of Ser118 or Y537 respectively, this event associated with ER transcriptional activation. These results suggest that MAPK or c-Src may be a common point of signal integration for c-ErbB2 and the ERα. It is possible that an increase in c-ErbB2/EGFR activity can activate the MAPK pathway either indirectly via a Shc/c-Src interaction or directly via Grb2, which can then activate the transcriptional activation of ERα resulting in a ligand independent estrogenic response. The activity of ERα can then activate the transcription of EGFR family members thereby perpetuating the cycle. The flexible nature of estrogen receptor signaling may thus explain why this receptor plays such an important role in both normal development and cancer, and provides an explanation as to why adjuvant therapies that involve antiestrogens are so successful.

While these signaling pathways have been shown to play a role in regulating mammary gland development, it is clear that spatial factors must also be considered (Figure 5.2). Specifically, the effect of estrogen on epithelial cell expansion during development is not autonomous in that cell-to-cell crosstalk in addition to receptor crosstalk is evident. A model for mammary gland development with the data presented in this thesis would include a stromally derived ERα signal that can be influenced by c-Src. The activation of ERα would induce the transcription of a number of ERE containing genes involved in ductal development during postnatal development. For example, the generation of EGF can signal in an autocrine fashion as well as to the adjacent epithelial cells, initiating ductal development and survival. Heterodimers of c-ErbB2 and EGFR may become activated (Sebastian et al., 1998) and initiate signals via c-Src which can then activate MAPK, ERα or both. Taken together, c-Src appears to play spatially compartmentalized roles, in the stroma and the epithelium, leading to the proper formation of mammary gland structures. At the intracellular level, c-Src within these cellular compartments regulate two receptor systems that play important roles in activating the signal transduction pathways required for mammary gland development. The elucidation of the mechanism whereby c-Src regulates both the c-ErbB2 receptor and

Figure 5.2 Molecular events that translate into stromal-epithelial interactions within the mammary gland.

Shown are some of the molecular pathways described in this thesis that may impinge on the stromal-epithelial interactions that are important in the development of the mammary gland. The current literature suggests that estrogen receptor function is indirect in that ERα containing stromal cells can define the archecture of the epithelial structure. The production of EGF on stromal cells has been suggested to stimulate the EGFR family found expressed on epithelial cells. This in turn activates signaling cascades that ultimately lead to transcriptional activation. The loss of c-Src in the mammary gland has been found to impinge on the function of two receptors that appear to play compartmentalized roles within the mammary gland. Loss of c-Src function can affect both ERα and c-ErbB2 function, the resulting phenotype leading to delays in mammary glanddevelopment.


c-ErbB2
EGFR
c-Src
E2/
ERα
EGF
EGFR
Stromal
Ductal architecture
EGF
c-ErbB2
EGFR
Epithelial
Epithelial cell proliferaion
and survival
c-Src
Ras/MAPK
ERα
EGFR
EGFR

ERα may help in the understanding of basic mammary gland development as well as shed light on the mechanisms of crosstalk that occurs during transformation and tumorigenesis.

CHAPTER 6

References

Alonso, G., Koegl, M., Mazurenko, N. and Courtneidge, S.A. (1995) Sequence requirements for binding of Src family tyrosine kinases to activated growth factor receptors. *J Biol Chem*, **270**, 9840-9848.

Alt, J.R., Cleveland, J.L., Hannink, M. and Diehl, J.A. (2000) Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. *Genes Dev*, **14**, 3102-3114.

Anderson, K.E., Coadwell, J., Stephens, L.R. and Hawkins, P.T. (1998) Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B. *Curr Biol*, **8**, 684-691.

Andrechek, E.R., Hardy, W.R., Siegel, P.M., Rudnicki, M.A., Cardiff, R.D. and Muller, W.J. (2000) Amplification of the neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis. *Proc Natl Acad Sci U S A*.

Andrulis, I.L., Bull, S.B., Blackstein, M.E., Sutherland, D., Mak, C., Sidlofsky, S., Pritzker, K.P., Hartwick, R.W., Hanna, W., Lickley, L., Wilkinson, R., Qizilbash, A., Ambus, U., Lipa, M., Weizel, H., Katz, A., Baida, M., Mariz, S., Stoik, G., Dacamara, P., Strongitharm, D., Geddie, W. and McCready, D. (1998) neu/erbB-2 amplification identifies a poor-prognosis group of women with node-negative breast cancer. Toronto Breast Cancer Study Group. *J Clin Oncol*, **16**, 1340-1349.

Antoniotti, S., Maggiora, P., Dati, C. and De Bortoli, M. (1992) Tamoxifen up-regulates c-erbB-2 expression in oestrogen-responsive breast cancer cells in vitro. *Eur J Cancer*, **28**, 318-321.

Aranda, A. and Pascual, A. (2001) Nuclear hormone receptors and gene expression. *Physiol Rev*, **81**, 1269-1304.

Arnold, S.F., Vorojeikina, D.P. and Notides, A.C. (1995) Phosphorylation of tyrosine 537 on the human estrogen receptor is required for binding to an estrogen response element. *J Biol Chem*, **270**, 30205-30212.

Aziz, N., Cherwinski, H. and McMahon, M. (1999) Complementation of defective colony-stimulating factor 1 receptor signaling and mitogenesis by Raf and v-Src. *Mol Cell Biol*, **19**, 1101-1115.

Bargmann, C.I., Hung, M.C. and Weinberg, R.A. (1986) Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. *Cell*, **45**, 649-657.

Bargmann, C.I. and Weinberg, R.A. (1988a) Increased tyrosine kinase activity associated with the protein encoded by the activated neu oncogene. *Proc Natl Acad Sci U S A*, **85**, 5394-5398.

Bargmann, C.I. and Weinberg, R.A. (1988b) Oncogenic activation of the neu-encoded receptor protein by point mutation and deletion. *Embo J*, 7, 2043-2052.

Basu, M., Biswas, R. and Das, M. (1984) 42,000-molecular weight EGF receptor has protein kinase activity. *Nature*, **311**, 477-480.

Bauknecht, T., Kohler, M., Janz, I. and Pfleiderer, A. (1989) The occurrence of epidermal growth factor receptors and the characterization of EGF-like factors in human ovarian, endometrial, cervical and breast cancer. EGF receptors and factors in gynecological carcinomas. *J Cancer Res Clin Oncol*, **115**, 193-199.

Beatson, G.T. (1896) On the treatment of inoperable cases of carcinoma of the mamma: Suggestions from a new method of treatment, with illustrative cases. *Lancet*, **ii**, 104-107.

Beerli, R.R., Wels, W. and Hynes, N.E. (1994) Autocrine inhibition of the epidermal growth factor receptor by intracellular expression of a single-chain antibody. *Biochem Biophys Res Commun*, **204**, 666-672.

Bellacosa, A., Chan, T.O., Ahmed, N.N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J. and Tsichlis, P. (1998) Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene*, **17**, 313-325.

Berry, M., Metzger, D. and Chambon, P. (1990) Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. *Embo J*, **9**, 2811-2818.

Bibbins, K.B., Boeuf, H. and Varmus, H.E. (1993) Binding of the Src SH2 domain to phosphopeptides is determined by residues in both the SH2 domain and the phosphopeptides. *Mol Cell Biol*, **13**, 7278-7287.

Billig, H., Furuta, I. and Hsueh, A.J. (1993) Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. *Endocrinology*, **133**, 2204-2212.

Biscardi, J.S., Maa, M.C., Tice, D.A., Cox, M.E., Leu, T.H. and Parsons, S.J. (1999) c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. *J Biol Chem*, **274**, 8335-8343.

Blake, R.A., Broome, M.A., Liu, X., Wu, J., Gishizky, M., Sun, L. and Courtneidge, S.A. (2000) SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. *Mol Cell Biol*, **20**, 9018-9027.

Bocchinfuso, W.P., Hively, W.P., Couse, J.F., Varmus, H.E. and Korach, K.S. (1999) A mouse mammary tumor virus-Wnt-1 transgene induces mammary gland hyperplasia and tumorigenesis in mice lacking estrogen receptor-alpha. *Cancer Res*, **59**, 1869-1876.

Bocchinfuso, W.P., Lindzey, J.K., Hewitt, S.C., Clark, J.A., Myers, P.H., Cooper, R. and Korach, K.S. (2000) Induction of mammary gland development in estrogen receptor-alpha knockout mice. *Endocrinology*, **141**, 2982-2994.

Bouton, A.H., Kanner, S.B., Vines, R.R., Wang, H.C., Gibbs, J.B. and Parsons, J.T. (1991) Transformation by pp60src or stimulation of cells with epidermal growth factor induces the stable association of tyrosine-phosphorylated cellular proteins with GTPase-activating protein. *Mol Cell Biol*, **11**, 945-953.

Boyce, B.F., Yoneda, T., Lowe, C., Soriano, P. and Mundy, G.R. (1992) Requirement of pp60c-src expression for osteoclasts to form ruffled borders and resorb bone in mice. *J Clin Invest*, **90**, 1622-1627.

Boyd, S. (1900) On oophorectomy in cancer of the breast. *Br Med J*, **ii**, 1161-1167.

Brandt, R., Eisenbrandt, R., Leenders, F., Zschiesche, W., Binas, B., Juergensen, C. and Theuring, F. (2000) Mammary gland specific hEGF receptor transgene expression induces neoplasia and inhibits differentiation. *Oncogene*, **19**, 2129-2137.

Brisken, C., Heineman, A., Chavarria, T., Elenbaas, B., Tan, J., Dey, S.K., McMahon, J.A., McMahon, A.P. and Weinberg, R.A. (2000) Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. *Genes Dev*, **14**, 650-654.

Britsch, S., Li, L., Kirchhoff, S., Theuring, F., Brinkmann, V., Birchmeier, C. and Riethmacher, D. (1998) The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. *Genes Dev*, **12**, 1825-1836.

Bromberg, J. and Darnell, J.E., Jr. (2000) The role of STATs in transcriptional control and their impact on cellular function. *Oncogene*, **19**, 2468-2473.

Broome, M.A. and Courtneidge, S.A. (2000) No requirement for src family kinases for PDGF signaling in fibroblasts expressing SV40 large T antigen. *Oncogene*, **19**, 2867-2869.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J. and Greenberg, M.E. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*, **96**, 857-868.

Buday, L. and Downward, J. (1993a) Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell*, **73**, 611-620.

Buday, L. and Downward, J. (1993b) Epidermal growth factor regulates the exchange rate of guanine nucleotides on p21ras in fibroblasts. *Mol Cell Biol*, **13**, 1903-1910.

Burke, C.L. and Stern, D.F. (1998) Activation of Neu (ErbB-2) mediated by disulfide bond-induced dimerization reveals a receptor tyrosine kinase dimer interface. *Mol Cell Biol*, **18**, 5371-5379.

Campbell, R.A., Bhat-Nakshatri, P., Patel, N.M., Constantinidou, D., Ali, S. and Nakshatri, H. (2001) Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. *J Biol Chem*, **276**, 9817-9824.

Cao, H., Bangalore, L., Bormann, B.J. and Stern, D.F. (1992) A subdomain in the transmembrane domain is necessary for p185neu* activation. *Embo J*, **11**, 923-932.

Cao, X., Tay, A., Guy, G.R. and Tan, Y.H. (1996) Activation and association of Stat3 with Src in v-Src-transformed cell lines. *Mol Cell Biol*, **16**, 1595-1603.

Carlomagno, C., Perrone, F., Gallo, C., De Laurentiis, M., Lauria, R., Morabito, A., Pettinato, G., Panico, L., D'Antonio, A., Bianco, A.R. and De Placido, S. (1996) c-erb B2 overexpression decreases the benefit of adjuvant tamoxifen in early-stage breast cancer without axillary lymph node metastases. *J Clin Oncol*, **14**, 2702-2708.

Carraway, K.L., 3rd and Cantley, L.C. (1994) A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. *Cell*, **78**, 5-8.

Castoria, G., Barone, M.V., Di Domenico, M., Bilancio, A., Ametrano, D., Migliaccio, A. and Auricchio, F. (1999) Non-transcriptional action of oestradiol and progestin triggers DNA synthesis. *Embo J*, **18**, 2500-2510.

Castoria, G., Migliaccio, A., Bilancio, A., Di Domenico, M., de Falco, A., Lombardi, M., Fiorentino, R., Varricchio, L., Barone, M.V. and Auricchio, F. (2001) PI3-kinase in concert with Src promotes the S-phase entry of oestradiol- stimulated MCF-7 cells. *Embo J*, **20**, 6050-6059.

Cavailles, V., Dauvois, S., Danielian, P.S. and Parker, M.G. (1994) Interaction of proteins with transcriptionally active estrogen receptors. *Proc Natl Acad Sci U S A*, **91**, 10009-10013.

Chambraud, B., Berry, M., Redeuilh, G., Chambon, P. and Baulieu, E.E. (1990) Several regions of human estrogen receptor are involved in the formation of receptor-heat shock protein 90 complexes. *J Biol Chem*, **265**, 20686-20691.

Chan, T.O., Rodeck, U., Chan, A.M., Kimmelman, A.C., Rittenhouse, S.E., Panayotou, G. and Tsichlis, P.N. (2002) Small GTPases and tyrosine kinases coregulate a molecular switch in the phosphoinositide 3-kinase regulatory subunit. *Cancer Cell*, **1**, 181-191.

Chang, C.M., Shu, H.K. and Kung, H.J. (1995) Disease specificity of kinase domains: the src-encoded catalytic domain converts erbB into a sarcoma oncogene. *Proc Natl Acad Sci U S A*, **92**, 3928-3932.

Chapman, R.S., Lourenco, P., Tonner, E., Flint, D., Selbert, S., Takeda, K., Akira, S., Clarke, A.R. and Watson, C.J. (2000) The role of Stat3 in apoptosis and mammary gland involution. Conditional deletion of Stat3. *Adv Exp Med Biol*, **480**, 129-138.

Chapman, R.S., Lourenco, P.C., Tonner, E., Flint, D.J., Selbert, S., Takeda, K., Akira, S., Clarke, A.R. and Watson, C.J. (1999) Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. *Genes Dev*, **13**, 2604-2616.

Clarke, R., Skaar, T.C., Bouker, K.B., Davis, N., Lee, Y.R., Welch, J.N. and Leonessa, F. (2001) Molecular and pharmacological aspects of antiestrogen resistance. *J Steroid Biochem Mol Biol*, **76**, 71-84.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M. and Hemmings, B.A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature*, **378**, 785-789.

Cross, F.R., Garber, E.A., Pellman, D. and Hanafusa, H. (1984) A short sequence in the p60src N terminus is required for p60src myristylation and membrane association and for cell transformation. *Mol Cell Biol*, **4**, 1834-1842.

Cunha, G.R. (1994) Role of mesenchymal-epithelial interactions in normal and abnormal development of the mammary gland and prostate. *Cancer*, **74**, 1030-1044.

Cunha, G.R., Young, P., Hom, Y.K., Cooke, P.S., Taylor, J.A. and Lubahn, D.B. (1997) Elucidation of a role for stromal steroid hormone receptors in mammary gland growth and development using tissue recombinants. *J Mammary Gland Biol Neoplasia*, **2**, 393-402.

Curtis Hewitt, S., Couse, J.F. and Korach, K.S. (2000) Estrogen receptor transcription and transactivation: Estrogen receptor knockout mice: what their phenotypes reveal about mechanisms of estrogen action. *Breast Cancer Res*, **2**, 345-352.

Daniel, C.W., Silberstein, G.B. and Strickland, P. (1987) Direct action of 17 beta-estradiol on mouse mammary ducts analyzed by sustained release implants and steroid autoradiography. *Cancer Res*, **47**, 6052-6057.

Daniel, C.W.a.S., G.D. (1987) Postnatal development of the rodent mammary gland. *In Mammary Gland: Development Regulation and Function (ed M.C. Neville and C.W. Daniel) pp.3-36*
New York : Plenum Press.

Dankort, D., Jeyabalan, N., Jones, N., Dumont, D.J. and Muller, W.J. (2001) Multiple ErbB-2/Neu Phosphorylation Sites Mediate Transformation through Distinct Effector Proteins. *J Biol Chem*, **276**, 38921-38928.

Dankort, D.L., Wang, Z., Blackmore, V., Moran, M.F. and Muller, W.J. (1997) Distinct tyrosine autophosphorylation sites negatively and positively modulate neu-mediated transformation. *Mol Cell Biol*, **17**, 5410-5425.

Dati, C., Antoniotti, S., Taverna, D., Perroteau, I. and De Bortoli, M. (1990) Inhibition of c-erbB-2 oncogene expression by estrogens in human breast cancer cells. *Oncogene*, **5**, 1001-1006.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. and Greenberg, M.E. (1997) Akt phosphorylation of BAD couples survival signals to the cell- intrinsic death machinery. *Cell*, **91**, 231-241.

de Beer, T., Carter, R.E., Lobel-Rice, K.E., Sorkin, A. and Overduin, M. (1998) Structure and Asn-Pro-Phe binding pocket of the Eps15 homology domain. *Science*, **281**, 1357-1360.

de Cremoux, P., Martin, E.C., Vincent-Salomon, A., Dieras, V., Barbaroux, C., Liva, S., Pouillart, P., Sastre-Garau, X. and Magdelenat, H. (1999) Quantitative PCR analysis of c-erb B-2 (HER2/neu) gene amplification and comparison with p185(HER2/neu) protein expression in breast cancer drill biopsies. *Int J Cancer*, **83**, 157-161.

De Placido, S., Carlomagno, C., De Laurentiis, M. and Bianco, A.R. (1998) c-erbB2 expression predicts tamoxifen efficacy in breast cancer patients. *Breast Cancer Res Treat*, **52**, 55-64.

DeCaprio, J.A., Ludlow, J.W., Figge, J., Shew, J.Y., Huang, C.M., Lee, W.H., Marsilio, E., Paucha, E. and Livingston, D.M. (1988) SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell*, **54**, 275-283.

DeClue, J.E., Zhang, K., Redford, P., Vass, W.C. and Lowy, D.R. (1991) Suppression of src transformation by overexpression of full-length GTPase-activating protein (GAP) or of the GAP C terminus. *Mol Cell Biol*, **11**, 2819-2825.

DeFriend, D.J., Howell, A., Nicholson, R.I., Anderson, E., Dowsett, M., Mansel, R.E., Blamey, R.W., Bundred, N.J., Robertson, J.F., Saunders, C. and et al. (1994) Investigation of a new pure antiestrogen (ICI 182780) in women with primary breast cancer. *Cancer Res*, **54**, 408-414.

Dhalluin, C., Yan, K., Plotnikova, O., Lee, K.W., Zeng, L., Kuti, M., Mujtaba, S., Goldfarb, M.P. and Zhou, M.M. (2000) Structural basis of SNT PTB domain interactions with distinct neurotrophic receptors. *Mol Cell*, **6**, 921-929.

Di Domenico, M., Castoria, G., Bilancio, A., Migliaccio, A. and Auricchio, F. (1996) Estradiol activation of human colon carcinoma-derived Caco-2 cell growth. *Cancer Res*, **56**, 4516-4521.

Di Fiore, P.P., Helin, K., Kraus, M.H., Pierce, J.H., Artrip, J., Segatto, O. and Bottaro, D.P. (1992) A single amino acid substitution is sufficient to modify the mitogenic properties of the epidermal growth factor receptor to resemble that of gp185erbB-2. *Embo J*, **11**, 3927-3933.

Di Fiore, P.P., Pierce, J.H., Fleming, T.P., Hazan, R., Ullrich, A., King, C.R., Schlessinger, J. and Aaronson, S.A. (1987a) Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells. *Cell*, **51**, 1063-1070.

Di Fiore, P.P., Pierce, J.H., Kraus, M.H., Segatto, O., King, C.R. and Aaronson, S.A. (1987b) erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. *Science*, **237**, 178-182.

Di Fiore, P.P., Segatto, O., Taylor, W.G., Aaronson, S.A. and Pierce, J.H. (1990) EGF receptor and erbB-2 tyrosine kinase domains confer cell specificity for mitogenic signaling. *Science*, **248**, 79-83.

Diehl, J.A., Cheng, M., Roussel, M.F. and Sherr, C.J. (1998) Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev*, **12**, 3499-3511.

Dikic, I., Schlessinger, J. and Lax, I. (1994) PC12 cells overexpressing the insulin receptor undergo insulin-dependent neuronal differentiation. *Curr Biol*, **4**, 702-708.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M.D. (1984) Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. *Nature*, **307**, 521-527.

Drebin, J.A., Stern, D.F., Link, V.C., Weinberg, R.A. and Greene, M.I. (1984) Monoclonal antibodies identify a cell-surface antigen associated with an activated cellular oncogene. *Nature*, **312**, 545-548.

Dubik, D. and Shiu, R.P. (1988) Transcriptional regulation of c-myc oncogene expression by estrogen in hormone-responsive human breast cancer cells. *J Biol Chem*, **263**, 12705-12708.

Earp, H.S., Dawson, T.L., Li, X. and Yu, H. (1995) Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research. *Breast Cancer Res Treat*, **35**, 115-132.

Eck, M.J., Shoelson, S.E. and Harrison, S.C. (1993) Recognition of a high-affinity phosphotyrosyl peptide by the Src homology-2 domain of p56lck. *Nature*, **362**, 87-91.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, **411**, 494-498.

Ellis, L., Clauser, E., Morgan, D.O., Edery, M., Roth, R.A. and Rutter, W.J. (1986) Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. *Cell*, **45**, 721-732.

Erpel, T., Alonso, G., Roche, S. and Courtneidge, S.A. (1996) The Src SH3 domain is required for DNA synthesis induced by platelet- derived growth factor and epidermal growth factor. *J Biol Chem*, **271**, 16807-16812.

Erpel, T., Superti-Furga, G. and Courtneidge, S.A. (1995) Mutational analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions. *Embo J*, **14**, 963-975.

Fantl, V., Stamp, G., Andrews, A., Rosewell, I. and Dickson, C. (1995) Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. *Genes Dev*, **9**, 2364-2372.

Fazioli, F., Kim, U.H., Rhee, S.G., Molloy, C.J., Segatto, O. and Di Fiore, P.P. (1991) The erbB-2 mitogenic signaling pathway: tyrosine phosphorylation of phospholipase C-gamma and GTPase-activating protein does not correlate with erbB-2 mitogenic potency. *Mol Cell Biol*, **11**, 2040-2048.

Fedorov, A.A., Fedorov, E., Gertler, F. and Almo, S.C. (1999) Structure of EVH1, a novel proline-rich ligand-binding module involved in cytoskeletal dynamics and neural function. *Nat Struct Biol*, **6**, 661-665.

Felder, S., Zhou, M., Hu, P., Urena, J., Ullrich, A., Chaudhuri, M., White, M., Shoelson, S.E. and Schlessinger, J. (1993) SH2 domains exhibit high-affinity binding to tyrosine-phosphorylated peptides yet also exhibit rapid dissociation and exchange. *Mol Cell Biol*, **13**, 1449-1455.

Feng, S., Chen, J.K., Yu, H., Simon, J.A. and Schreiber, S.L. (1994) Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3-ligand interactions. *Science*, **266**, 1241-1247.

Ferguson, K.M., Darling, P.J., Mohan, M.J., Macatee, T.L and Lemmon, M.A. (2000) Extracellular domains drive homo- but not hetero-dimerization of erbB receptors. *Embo J.* **19**, 4632-4643.

Fisher, C.R., Graves, K.H., Parlow, A.F. and Simpson, E.R. (1998) Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proc Natl Acad Sci U S A*, **95**, 6965-6970.

Gallego, M.I., Binart, N., Robinson, G.W., Okagaki, R., Coschigano, K.T., Perry, J., Kopchick, J.J., Oka, T., Kelly, P.A. and Hennighausen, L. (2001) Prolactin, growth hormone, and epidermal growth factor activate Stat5 in different compartments of mammary tissue and exert different and overlapping developmental effects. *Dev Biol*, **229**, 163-175.

Garcia de Palazzo, I.E., Adams, G.P., Sundareshan, P., Wong, A.J., Testa, J.R., Bigner, D.D. and Weiner, L.M. (1993) Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. *Cancer Res*, **53**, 3217-3220.

Giguere V, Yang N, Segui P, Evans RM. (1988) Identification of a new class of steroid hormone receptors. Nature. Jan 7;331(6151):91-4.

Gotoh, N., Tojo, A., Hino, M., Yazaki, Y. and Shibuya, M. (1992) A highly conserved tyrosine residue at codon 845 within the kinase domain is not required for the transforming activity of human epidermal growth factor receptor. *Biochem Biophys Res Commun*, **186**, 768-774.

Gould, K.L., Woodgett, J.R., Cooper, J.A., Buss, J.E., Shalloway, D. and Hunter, T. (1985) Protein kinase C phosphorylates pp60src at a novel site. *Cell*, **42**, 849-857.

Gouon-Evans V, Rothenberg ME, Pollard JW. (2000) Postnatal mammary gland development requires macrophages and eosinophils.Development. Jun;127(11):2269-82.

Graus-Porta, D., Beerli, R.R., Daly, J.M. and Hynes, N.E. (1997) ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *Embo J*, **16**, 1647-1655.

Greene, L.A. and Tischler, A.S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci U S A*, **73**, 2424-2428.

Grunt, T.W., Saceda, M., Martin, M.B., Lupu, R., Dittrich, E., Krupitza, G., Harant, H., Huber, H. and Dittrich, C. (1995) Bidirectional interactions between the estrogen receptor and the cerbB- 2 signaling pathways: heregulin inhibits estrogenic effects in breast cancer cells. *Int J Cancer*, **63**, 560-567.

Gullick, W.J., Bottomley, A.C., Lofts, F.J., Doak, D.G., Mulvey, D., Newman, R., Crumpton, M.J., Sternberg, M.J. and Campbell, I.D. (1992) Three dimensional structure of the transmembrane region of the proto- oncogenic and oncogenic forms of the neu protein. *Embo J*, **11**, 43-48.

Gullick, W.J., Love, S.B., Wright, C., Barnes, D.M., Gusterson, B., Harris, A.L. and Altman, D.G. (1991) c-erbB-2 protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. *Br J Cancer*, **63**, 434-438.

Guy, C.T., Cardiff, R.D. and Muller, W.J. (1996) Activated neu induces rapid tumor progression. *J Biol Chem*, **271**, 7673-7678.

Guy, C.T., Muthuswamy, S.K., Cardiff, R.D., Soriano, P. and Muller, W.J. (1994a) Activation of the c-Src tyrosine kinase is required for the induction of mammary tumors in transgenic mice. *Genes Dev*, **8**, 23-32.

Guy, C.T., Webster, M.A., Schaller, M., Parsons, T.J., Cardiff, R.D. and Muller, W.J. (1992) Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci U S A*, **89**, 10578-10582.

Guy, P.M., Platko, J.V., Cantley, L.C., Cerione, R.A. and Carraway, K.L., 3rd. (1994b) Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. *Proc Natl Acad Sci U S A*, **91**, 8132-8136.

Hanahan, D. and Weinberg, R.A. (2000) The hallmarks of cancer. *Cell*, **100**, 57-70.

Hansen, K., Johnell, M., Siegbahn, A., Rorsman, C., Engstrom, U., Wernstedt, C., Heldin, C.H. and Ronnstrand, L. (1996) Mutation of a Src phosphorylation site in the

PDGF beta-receptor leads to increased PDGF-stimulated chemotaxis but decreased mitogenesis. *Embo J*, **15**, 5299-5313.

Harris, A.L., Nicholson, S., Sainsbury, R., Wright, C. and Farndon, J. (1992) Epidermal growth factor receptor and other oncogenes as prognostic markers. *J Natl Cancer Inst Monogr*, **11**, 181-187.

Haslam, S.Z. and Nummy, K.A. (1992) The ontogeny and cellular distribution of estrogen receptors in normal mouse mammary gland. *J Steroid Biochem Mol Biol*, **42**, 589-595.

Hazan, R., Margolis, B., Dombalagian, M., Ullrich, A., Zilberstein, A. and Schlessinger, J. (1990) Identification of autophosphorylation sites of HER2/neu. *Cell Growth Differ*, **1**, 3-7.

Heasley, L.E. and Johnson, G.L. (1992) The beta-PDGF receptor induces neuronal differentiation of PC12 cells. *Mol Biol Cell*, **3**, 545-553.

Hennighausen, L. and Robinson, G.W. (1998) Think globally, act locally: the making of a mouse mammary gland. *Genes Dev*, **12**, 449-455.

Hennighausen, L. and Robinson, G.W. (2001) Signaling pathways in mammary gland development. *Dev Cell*, **1**, 467-475.

Hewitt, S.C., Bocchinfuso, W.P., Zhai, J., Harrell, C., Koonce, L., Clark, J., Myers, P. and Korach, K.S. (2002) Lack of ductal development in the absence of functional estrogen receptor alpha delays mammary tumor formation induced by transgenic expression of ErbB2/neu. *Cancer Res*, **62**, 2798-2805.

Hirota, Y., Kato, J. and Takeya, T. (1988) Substitution of Ser-17 of pp60c-src: biological and biochemical characterization in chicken embryo fibroblasts. *Mol Cell Biol*, **8**, 1826-1830.

Hisamoto, K., Ohmichi, M., Kurachi, H., Hayakawa, J., Kanda, Y., Nishio, Y., Adachi, K., Tasaka, K., Miyoshi, E., Fujiwara, N., Taniguchi, N. and Murata, Y. (2001) Estrogen induces the Akt-dependent activation of endothelial nitric- oxide synthase in vascular endothelial cells. *J Biol Chem*, **276**, 3459-3467.

Houston, S.J., Plunkett, T.A., Barnes, D.M., Smith, P., Rubens, R.D. and Miles, D.W. (1999) Overexpression of c-erbB2 is an independent marker of resistance to endocrine therapy in advanced breast cancer. *Br J Cancer*, **79**, 1220-1226.

Hubbard, S.R., Mohammadi, M. and Schlessinger, J. (1998) Autoregulatory mechanisms in protein-tyrosine kinases. *J Biol Chem*, **273**, 11987-11990.

Huff, K., End, D. and Guroff, G. (1981) Nerve growth factor-induced alteration in the response of PC12 pheochromocytoma cells to epidermal growth factor. *J Cell Biol*, **88**, 189-198.

Hulka, B.S. and Stark, S.T. (1995) Breast cancer: Cause and prevention. *Lancet*, **346**, 883-887.

Hunter, T. and Sefton, B.M. (1980) Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc Natl Acad Sci U S A*, **77**, 1311-1315.

Hurley, J.H. and Meyer, T. (2001) Subcellular targeting by membrane lipids. *Curr Opin Cell Biol*, **13**, 146-152.

Hutchinson, J., Jin, J., Cardiff, R.D., Woodgett, J.R. and Muller, W.J. (2001) Activation of Akt (protein kinase B) in mammary epithelium provides a critical cell survival signal required for tumor progression. *Mol Cell Biol*, **21**, 2203-2212.

Imamoto, A. and Soriano, P. (1993) Disruption of the csk gene, encoding a negative regulator of Src family tyrosine kinases, leads to neural tube defects and embryonic lethality in mice. *Cell*, **73**, 1117-1124.

Itakura, Y., Sasano, H., Shiga, C., Furukawa, Y., Shiga, K., Mori, S. and Nagura, H. (1994) Epidermal growth factor receptor overexpression in esophageal carcinoma. An immunohistochemical study correlated with clinicopathologic findings and DNA amplification. *Cancer*, **74**, 795-804.

Jiang, G. and Hunter, T. (1999) Receptor signaling: when dimerization is not enough. *Curr Biol*, **9**, R568-571.

Kaech, S., Schnierle, B., Wyss, A. and Ballmer-Hofer, K. (1993) Myristylation and amino-terminal phosphorylation are required for activation of pp60c-src during mitosis. *Oncogene*, **8**, 575-581.

Kaplan, J.M., Mardon, G., Bishop, J.M. and Varmus, H.E. (1988) The first seven amino acids encoded by the v-src oncogene act as a myristylation signal: lysine 7 is a critical determinant. *Mol Cell Biol*, **8**, 2435-2441.

Kaplan, K.B., Swedlow, J.R., Morgan, D.O. and Varmus, H.E. (1995) c-Src enhances the spreading of src-/- fibroblasts on fibronectin by a kinase-independent mechanism. *Genes Dev*, **9**, 1505-1517.

Karunagaran, D., Tzahar, E., Beerli, R.R., Chen, X., Graus-Porta, D., Ratzkin, B.J., Seger, R., Hynes, N.E. and Yarden, Y. (1996) ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *Embo J*, **15**, 254-264.

Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H. and et al. (1995) Activation of the estrogen receptor through phosphorylation by mitogen- activated protein kinase. *Science*, **270**, 1491-1494.

Kimber, M.S., Nachman, J., Cunningham, A.M., Gish, G.D., Pawson, T. and Pai, E.F. (2000) Structural basis for specificity switching of the Src SH2 domain. *Mol Cell*, **5**, 1043-1049.

Klinghoffer, R.A., Sachsenmaier, C., Cooper, J.A. and Soriano, P. (1999) Src family kinases are required for integrin but not PDGFR signal transduction. *Embo J*, **18**, 2459-2471.

Kmiecik, T.E. and Shalloway, D. (1987) Activation and suppression of pp60c-src transforming ability by mutation of its primary sites of tyrosine phosphorylation. *Cell*, **49**, 65-73.

Kobayashi, Y., Kitamoto, T., Masuhiro, Y., Watanabe, M., Kase, T., Metzger, D., Yanagisawa, J. and Kato, S. (2000) p300 mediates functional synergism between AF-1 and AF-2 of estrogen receptor alpha and beta by interacting directly with the N-terminal A/B domains. *J Biol Chem*, **275**, 15645-15651.

Kokai, Y., Myers, J.N., Wada, T., Brown, V.I., LeVea, C.M., Davis, J.G., Dobashi, K. and Greene, M.I. (1989) Synergistic interaction of p185c-neu and the EGF receptor leads to transformation of rodent fibroblasts. *Cell*, **58**, 287-292.

Kong, Y.Y., Yoshida, H., Sarosi, I., Tan, H.L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A.J., Van, G., Itie, A., Khoo, W., Wakeham, A., Dunstan, C.R., Lacey, D.L., Mak, T.W., Boyle, W.J. and Penninger, J.M. (1999) OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature*, **397**, 315-323.

Korach, K.S., Couse, J.F., Curtis, S.W., Washburn, T.F., Lindzey, J., Kimbro, K.S., Eddy, E.M., Migliaccio, S., Snedeker, S.M., Lubahn, D.B., Schomberg, D.W. and Smith, E.P. (1996) Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. *Recent Prog Horm Res*, **51**, 159-186.

Kremer, N.E., D'Arcangelo, G., Thomas, S.M., DeMarco, M., Brugge, J.S. and Halegoua, S. (1991) Signal transduction by nerve growth factor and fibroblast growth factor in PC12 cells requires a sequence of src and ras actions. *J Cell Biol*, **115**, 809-819.

Kumar, T.R., Wang, Y., Lu, N. and Matzuk, M.M. (1997) Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet*, **15**, 201-204.

Kwan, H., Pecenka, V., Tsukamoto, A., Parslow, T.G., Guzman, R., Lin, T.P., Muller, W.J., Lee, F.S., Leder, P. and Varmus, H.E. (1992) Transgenes expressing the Wnt-1 and int-2 proto-oncogenes cooperate during mammary carcinogenesis in doubly transgenic mice. *Mol Cell Biol*, **12**, 147-154.

Ladbury, J.E., Lemmon, M.A., Zhou, M., Green, J., Botfield, M.C. and Schlessinger, J. (1995) Measurement of the binding of tyrosyl phosphopeptides to SH2 domains: a reappraisal. *Proc Natl Acad Sci U S A*, **92**, 3199-3203.

Laudano, A.P. and Buchanan, J.M. (1986) Phosphorylation of tyrosine in the carboxyl-terminal tryptic peptide of pp60c-src. *Proc Natl Acad Sci U S A*, **83**, 892-896.

Lemmon, M.A., Bu, Z., Ladbury, J.E., Zhou, M., Pinchasi, D., Lax, I., Engelman, D.M. and Schlessinger, J. (1997) Two EGF molecules contribute additively to stabilization of the EGFR dimer. *Embo J*, **16**, 281-294.

Lenferink, A.E., Simpson, J.F., Shawver, L.K., Coffey, R.J., Forbes, J.T. and Arteaga, C.L. (2000) Blockade of the epidermal growth factor receptor tyrosine kinase suppresses tumorigenesis in MMTV/Neu + MMTV/TGF-alpha bigenic mice. *Proc Natl Acad Sci U S A*, **97**, 9609-9614.

Leung, B.S. (1987) Mode of estrogen action on cell proliferation in CAMA-1 cells: III. Effect of antiestrogen. *Anticancer Res*, **7**, 219-225.

Levine, A.J. and Momand, J. (1990) Tumor suppressor genes: the p53 and retinoblastoma sensitivity genes and gene products. *Biochim Biophys Acta*, **1032**, 119-136.

Li, M., Liu, X., Robinson, G., Bar-Peled, U., Wagner, K.U., Young, W.S., Hennighausen, L. and Furth, P.A. (1997) Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. *Proc Natl Acad Sci U S A*, **94**, 3425-3430.

Lin, T.H., Aplin, A.E., Shen, Y., Chen, Q., Schaller, M., Romer, L., Aukhil, I. and Juliano, R.L. (1997) Integrin-mediated activation of MAP kinase is independent of FAK: evidence for dual integrin signaling pathways in fibroblasts. *J Cell Biol*, **136**, 1385-1395.

Lipponen, P. and Eskelinen, M. (1994) Expression of epidermal growth factor receptor in bladder cancer as related to established prognostic factors, oncoprotein (c-erbB-2, p53) expression and long-term prognosis. *Br J Cancer*, **69**, 1120-1125.

Liu, E., Thor, A., He, M., Barcos, M., Ljung, B.M. and Benz, C. (1992) The HER2 (c-erbB-2) oncogene is frequently amplified in in situ carcinomas of the breast. *Oncogene*, **7**, 1027-1032.

Liu, S., Liu, W., Jakubczak, J.L., Erexson, G.L., Tindall, K.R., Chan, R., Muller, W.J., Adhya, S., Garges, S. and Merlino, G. (2002) Genetic instability favoring transversions associated with ErbB2-induced mammary tumorigenesis. *Proc Natl Acad Sci U S A*, **99**, 3770-3775.

Liu, X., Brodeur, S.R., Gish, G., Songyang, Z., Cantley, L.C., Laudano, A.P. and Pawson, T. (1993) Regulation of c-Src tyrosine kinase activity by the Src SH2 domain. *Oncogene*, **8**, 1119-1126.

Liu, X., Robinson, G.W., Wagner, K.U., Garrett, L., Wynshaw-Boris, A. and Hennighausen, L. (1997) Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev*, **11**, 179-186.

Lonard, D.M., Nawaz, Z., Smith, C.L. and O'Malley, B.W. (2000) The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. *Mol Cell*, **5**, 939-948.

Lovekin, C., Ellis, I.O., Locker, A., Robertson, J.F., Bell, J., Nicholson, R., Gullick, W.J., Elston, C.W. and Blamey, R.W. (1991) c-erbB-2 oncoprotein expression in primary and advanced breast cancer [published erratum appears in Br J Cancer 1991 Jul;64(1):202]. *Br J Cancer*, **63**, 439-443.

Lowe, C., Yoneda, T., Boyce, B.F., Chen, H., Mundy, G.R. and Soriano, P. (1993) Osteopetrosis in Src-deficient mice is due to an autonomous defect of osteoclasts. *Proc Natl Acad Sci U S A*, **90**, 4485-4489.

Lubahn, D.B., Moyer, J.S., Golding, T.S., Couse, J.F., Korach, K.S. and Smithies, O. (1993) Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci U S A*, **90**, 11162-11166.

Luetteke, N.C., Phillips, H.K., Qiu, T.H., Copeland, N.G., Earp, H.S., Jenkins, N.A. and Lee, D.C. (1994) The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. *Genes Dev*, **8**, 399-413.

Luttrell, D.K., Luttrell, L.M. and Parsons, S.J. (1988) Augmented mitogenic responsiveness to epidermal growth factor in murine fibroblasts that overexpress pp60c-src. *Mol Cell Biol*, **8**, 497-501.

Maa, M.C., Leu, T.H., McCarley, D.J., Schatzman, R.C. and Parsons, S.J. (1995) Potentiation of epidermal growth factor receptor-mediated oncogenesis by c-Src:

implications for the etiology of multiple human cancers. *Proc Natl Acad Sci U S A*, **92**, 6981-6985.

MacGregor, J.I. and Jordan, V.C. (1998) Basic guide to the mechanisms of antiestrogen action. *Pharmacol Rev*, **50**, 151-196.

Macias, M.J., Hyvonen, M., Baraldi, E., Schultz, J., Sudol, M., Saraste, M. and Oschkinat, H. (1996) Structure of the WW domain of a kinase-associated protein complexed with a proline-rich peptide. *Nature*, **382**, 646-649.

Marengere, L.E., Songyang, Z., Gish, G.D., Schaller, M.D., Parsons, J.T., Stern, M.J., Cantley, L.C. and Pawson, T. (1994) SH2 domain specificity and activity modified by a single residue. *Nature*, **369**, 502-505.

Marx, J. (2000) Interfering with gene expression. *Science*, **288**, 1370-1372.

Matsui, Y., Halter, S.A., Holt, J.T., Hogan, B.L. and Coffey, R.J. (1990) Development of mammary hyperplasia and neoplasia in MMTV-TGF alpha transgenic mice. *Cell*, **61**, 1147-1155.

Merlino, G.T., Xu, Y.H., Ishii, S., Clark, A.J., Semba, K., Toyoshima, K., Yamamoto, T. and Pastan, I. (1984) Amplification and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. *Science*, **224**, 417-419.

Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E. and Auricchio, F. (1996) Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol- receptor complex in MCF-7 cells. *Embo J*, **15**, 1292-1300.

Migliaccio, A., Pagano, M. and Auricchio, F. (1993) Immediate and transient stimulation of protein tyrosine phosphorylation by estradiol in MCF-7 cells. *Oncogene*, **8**, 2183-2191.

Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W., Beato, M. and Auricchio, F. (1998) Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. *Embo J*, **17**, 2008-2018.

Miller, M., Ginalski, K., Lesyng, B., Nakaigawa, N., Schmidt, L. and Zbar, B. (2001) Structural basis of oncogenic activation caused by point mutations in the kinase domain of the MET proto-oncogene: modeling studies. *Proteins*, **44**, 32-43.

Mixner, J.a.C.T. (1942) Role of estrogen in the stimulation of lobule-alveolar growth by progesterone and by the mammogenic lobule-alveolar growth factor of the anterior patuitary. *Endocrinology*, **30**, 591-597.

Moarefi, I., LaFevre-Bernt, M., Sicheri, F., Huse, M., Lee, C.H., Kuriyan, J. and Miller, W.T. (1997) Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. *Nature*, **385**, 650-653.

Mohammadi, M., Dikic, I., Sorokin, A., Burgess, W.H., Jaye, M. and Schlessinger, J. (1996) Identification of six novel autophosphorylation sites on fibroblast growth factor receptor 1 and elucidation of their importance in receptor activation and signal transduction. *Mol Cell Biol*, **16**, 977-989.

Morais Cabral, J.H., Petosa, C., Sutcliffe, M.J., Raza, S., Byron, O., Poy, F., Marfatia, S.M., Chishti, A.H. and Liddington, R.C. (1996) Crystal structure of a PDZ domain. *Nature*, **382**, 649-652.

Mori, S., Ronnstrand, L., Yokote, K., Engstrom, A., Courtneidge, S.A., Claesson-Welsh, L. and Heldin, C.H. (1993) Identification of two juxtamembrane autophosphorylation sites in the PDGF beta-receptor; involvement in the interaction with Src family tyrosine kinases. *Embo J*, **12**, 2257-2264.

Moro, L., Venturino, M., Bozzo, C., Silengo, L., Altruda, F., Beguinot, L., Tarone, G. and Defilippi, P. (1998) Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival. *Embo J*, **17**, 6622-6632.

Morris, J.K., Lin, W., Hauser, C., Marchuk, Y., Getman, D. and Lee, K.F. (1999) Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development [In Process Citation]. *Neuron*, **23**, 273-283.

Muller, W.J., Arteaga, C.L., Muthuswamy, S.K., Siegel, P.M., Webster, M.A., Cardiff, R.D., Meise, K.S., Li, F., Halter, S.A. and Coffey, R.J. (1996) Synergistic interaction of the Neu proto-oncogene product and transforming growth factor alpha in the mammary epithelium of transgenic mice. *Mol Cell Biol*, **16**, 5726-5736.

Muller, W.J., Sinn, E., Pattengale, P.K., Wallace, R. and Leder, P. (1988) Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell*, **54**, 105-115.

Musgrove, E.A., Sarcevic, B. and Sutherland, R.L. (1996) Inducible expression of cyclin D1 in T-47D human breast cancer cells is sufficient for Cdk2 activation and pRB hyperphosphorylation. *J Cell Biochem*, **60**, 363-378.

Muthuswamy, S.K., Li, D., Lelievre, S., Bissell, M.J. and Brugge, J.S. (2001) ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nat Cell Biol*, **3**, 785-792.

Muthuswamy, S.K. and Muller, W.J. (1994) Activation of the Src family of tyrosine kinases in mammary tumorigenesis. *Adv Cancer Res*, **64**, 111-123.

Muthuswamy, S.K. and Muller, W.J. (1995) Direct and specific interaction of c-Src with Neu is involved in signaling by the epidermal growth factor receptor. *Oncogene*, **11**, 271-279.

Muthuswamy, S.K., Siegel, P.M., Dankort, D.L., Webster, M.A. and Muller, W.J. (1994) Mammary tumors expressing the neu proto-oncogene possess elevated c-Src tyrosine kinase activity. *Mol Cell Biol*, **14**, 735-743.

Naski, M.C., Wang, Q., Xu, J. and Ornitz, D.M. (1996) Graded activation of fibroblast growth factor receptor 3 by mutations causing achondroplasia and thanatophoric dysplasia. *Nat Genet*, **13**, 233-237.

Nawaz, Z., Lonard, D.M., Dennis, A.P., Smith, C.L. and O'Malley, B.W. (1999) Proteasome-dependent degradation of the human estrogen receptor. *Proc Natl Acad Sci U S A*, **96**, 1858-1862.

Newby, J.C., Johnston, S.R., Smith, I.E. and Dowsett, M. (1997) Expression of epidermal growth factor receptor and c-erbB2 during the development of tamoxifen resistance in human breast cancer. *Clin Cancer Res*, **3**, 1643-1651.

Newman, S.P., Bates, N.P., Vernimmen, D., Parker, M.G. and Hurst, H.C. (2000) Cofactor competition between the ligand-bound oestrogen receptor and an intron 1 enhancer leads to oestrogen repression of ERBB2 expression in breast cancer. *Oncogene*, **19**, 490-497.

Niemann, C., Brinkmann, V., Spitzer, E., Hartmann, G., Sachs, M., Naundorf, H. and Birchmeier, W. (1998) Reconstitution of mammary gland development in vitro: requirement of c- met and c-erbB2 signaling for branching and alveolar morphogenesis. *J Cell Biol*, **143**, 533-545.

Niemeyer, C.C., Spencer-Dene, B., Wu, J.X. and Adamson, E.D. (1999) Preneoplastic mammary tumor markers: Cripto and Amphiregulin are overexpressed in hyperplastic stages of tumor progression in transgenic mice. *Int J Cancer*, **81**, 588-591.

Normanno, N., Ciardiello, F., Brandt, R. and Salomon, D.S. (1994) Epidermal growth factor-related peptides in the pathogenesis of human breast cancer. *Breast Cancer Res Treat*, **29**, 11-27.

Ohgaki, H., Vogeley, K.T., Kleihues, P. and Wechsler, W. (1993) neu mutations and loss of normal allele in schwannomas induced by N- ethyl-N-nitrosourea in rats. *Cancer Lett*, **70**, 45-50.

Ohmichi, M., Pang, L., Ribon, V. and Saltiel, A.R. (1993) Divergence of signaling pathways for insulin in PC-12 pheochromocytoma cells. *Endocrinology*, **133**, 46-56.

Okada, M., Howell, B.W., Broome, M.A. and Cooper, J.A. (1993) Deletion of the SH3 domain of Src interferes with regulation by the phosphorylated carboxyl-terminal tyrosine. *J Biol Chem*, **268**, 18070-18075.

Olayioye, M.A., Graus-Porta, D., Beerli, R.R., Rohrer, J., Gay, B. and Hynes, N.E. (1998) ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner. *Mol Cell Biol*, **18**, 5042-5051.

Onate, S.A., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science*, **270**, 1354-1357.

Osborne, C.K. (1998) Tamoxifen in the treatment of breast cancer. *N Engl J Med*, **339**, 1609-1618.

Osborne, C.K. and Fuqua, S.A. (1994) Mechanisms of tamoxifen resistance. *Breast Cancer Res Treat*, **32**, 49-55.

Osherov, N. and Levitzki, A. (1994) Epidermal-growth-factor-dependent activation of the src-family kinases. *Eur J Biochem*, **225**, 1047-1053.

Ottenhoff-Kalff, A.E., Rijksen, G., van Beurden, E.A., Hennipman, A., Michels, A.A. and Staal, G.E. (1992) Characterization of protein tyrosine kinases from human breast cancer: involvement of the c-src oncogene product. *Cancer Res*, **52**, 4773-4778.

Pandit, S.D., Donis-Keller, H., Iwamoto, T., Tomich, J.M. and Pike, L.J. (1996) The multiple endocrine neoplasia type 2B point mutation alters long- term regulation and

enhances the transforming capacity of the epidermal growth factor receptor. *J Biol Chem*, **271**, 5850-5858.

Paterson, M.C., Dietrich, K.D., Danyluk, J., Paterson, A.H., Lees, A.W., Jamil, N., Hanson, J., Jenkins, H., Krause, B.E., McBlain, W.A. and et al. (1991) Correlation between c-erbB-2 amplification and risk of recurrent disease in node-negative breast cancer. *Cancer Res*, **51**, 556-567.

Pawson, T. (1994) SH2 and SH3 domains in signal transduction. *Adv Cancer Res*, **64**, 87-110.

Pawson, T. and Nash, P. (2000) Protein-protein interactions define specificity in signal transduction. *Genes Dev*, **14**, 1027-1047.

Payne, G., Shoelson, S.E., Gish, G.D., Pawson, T. and Walsh, C.T. (1993) Kinetics of p56lck and p60src Src homology 2 domain binding to tyrosine- phosphorylated peptides determined by a competition assay or surface plasmon resonance. *Proc Natl Acad Sci U S A*, **90**, 4902-4906.

Perantoni, A.O., Rice, J.M., Reed, C.D., Watatani, M. and Wenk, M.L. (1987) Activated neu oncogene sequences in primary tumors of the peripheral nervous system induced in rats by transplacental exposure to ethylnitrosourea. *Proc Natl Acad Sci U S A*, **84**, 6317-6321.

Pestell, R.G., Albanese, C., Reutens, A.T., Segall, J.E., Lee, R.J. and Arnold, A. (1999) The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation. *Endocr Rev*, **20**, 501-534.

Phippard, D.J., Weber-Hall, S.J., Sharpe, P.T., Naylor, M.S., Jayatalake, H., Maas, R., Woo, I., Roberts-Clark, D., Francis-West, P.H., Liu, Y.H., Maxson, R., Hill, R.E. and Dale, T.C. (1996) Regulation of Msx-1, Msx-2, Bmp-2 and Bmp-4 during foetal and postnatal mammary gland development. *Development*, **122**, 2729-2737.

Plopper, G.E., McNamee, H.P., Dike, L.E., Bojanowski, K. and Ingber, D.E. (1995) Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. *Mol Biol Cell*, **6**, 1349-1365.

Posner, B.I., Faure, R., Burgess, J.W., Bevan, A.P., Lachance, D., Zhang-Sun, G., Fantus, I.G., Ng, J.B., Hall, D.A., Lum, B.S. and et al. (1994) Peroxovanadium compounds. A new class of potent phosphotyrosine phosphatase inhibitors which are insulin mimetics. *J Biol Chem*, **269**, 4596-4604.

Prall, O.W., Rogan, E.M., Musgrove, E.A., Watts, C.K. and Sutherland, R.L. (1998) c-Myc or cyclin D1 mimics estrogen effects on cyclin E-Cdk2 activation and cell cycle reentry. *Mol Cell Biol*, **18**, 4499-4508.

Prall, O.W.J., Sarcevic, B., Musgrove, E.A., Watts, C.K.W. and Sutherland, R.L. (1997) Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E- Cdk2. *J Biol Chem*, **272**, 10882-10894.

Prigent, S.A., Nagane, M., Lin, H., Huvar, I., Boss, G.R., Feramisco, J.R., Cavenee, W.K. and Huang, H.S. (1996) Enhanced tumorigenic behavior of glioblastoma cells expressing a truncated epidermal growth factor receptor is mediated through the Ras-Shc-Grb2 pathway. *J Biol Chem*, **271**, 25639-25645.

Qian, X., O'Rourke, D.M., Fei, Z., Zhang, H.T., Kao, C.C. and Greene, M.I. (1999) Domain-specific interactions between the p185(neu) and epidermal growth factor receptor kinases determine differential signaling outcomes. *J Biol Chem*, **274**, 574-583.

Rabin, S.J., Cleghon, V. and Kaplan, D.R. (1993) SNT, a differentiation-specific target of neurotrophic factor-induced tyrosine kinase activity in neurons and PC12 cells. *Mol Cell Biol*, **13**, 2203-2213.

Richer, J.K., Lange, C.A., Manning, N.G., Owen, G., Powell, R. and Horwitz, K.B. (1998) Convergence of progesterone with growth factor and cytokine signaling in breast cancer. Progesterone receptors regulate signal transducers and activators of transcription expression and activity. *J Biol Chem*, **273**, 31317-31326.

Riese, D.J., 2nd and Stern, D.F. (1998) Specificity within the EGF family/ErbB receptor family signaling network. *Bioessays*, **20**, 41-48.

Riese, D.J., Kim, E.D., Elenius, K., Buckley, S., Klagsbrun, M., Plowman, G.D. and Stern, D.F. (1996) The epidermal growth factor receptor couples transforming growth factor- alpha, heparin-binding epidermal growth factor-like factor, and amphiregulin to Neu, ErbB-3, and ErbB-4. *J Biol Chem*, **271**, 20047-20052.

Risma, K.A., Clay, C.M., Nett, T.M., Wagner, T., Yun, J. and Nilson, J.H. (1995) Targeted overexpression of luteinizing hormone in transgenic mice leads to infertility, polycystic ovaries, and ovarian tumors. *Proc Natl Acad Sci U S A*, **92**, 1322-1326.

Roche, S., Alonso, G., Kazlauskas, A., Dixit, V.M., Courtneidge, S.A. and Pandey, A. (1998) Src-like adaptor protein (Slap) is a negative regulator of mitogenesis. *Curr Biol*, **8**, 975-978.

Roche, S., Fumagalli, S. and Courtneidge, S.A. (1995a) Requirement for Src family protein tyrosine kinases in G2 for fibroblast cell division. *Science*, **269**, 1567-1569.

Roche, S., Koegl, M., Barone, M.V., Roussel, M.F. and Courtneidge, S.A. (1995b) DNA synthesis induced by some but not all growth factors requires Src family protein tyrosine kinases. *Mol Cell Biol*, **15**, 1102-1109.

Rotin, D., Margolis, B., Mohammadi, M., Daly, R.J., Daum, G., Li, N., Fischer, E.H., Burgess, W.H., Ullrich, A. and Schlessinger, J. (1992) SH2 domains prevent tyrosine dephosphorylation of the EGF receptor: identification of Tyr992 as the high-affinity binding site for SH2 domains of phospholipase C gamma. *Embo J*, **11**, 559-567.

Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. and Bowtell, D. (1993) The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1 [see comments]. *Nature*, **363**, 83-85.

Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P.G. and et al. (1992) Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature*, **360**, 689-692.

Ruff, M., Gangloff, M., Wurtz, J.M. and Moras, D. (2000) Estrogen receptor transcription and transactivation: Structure-function relationship in DNA- and ligand-binding domains of estrogen receptors. *Breast Cancer Res*, **2**, 353-359.

Sebastian J, Richards RG, Walker MP, Wiesen JF, Werb Z, Derynck R, Hom YK, Cunha GR, DiAugustine RP. (1998) Activation and function of the epidermal growth

factor receptor and erbB-2 during mammary gland morphogenesis.Cell Growth Differ. 1998 Sep;9(9):777-85.

Sabbagh, W., Flatauer, L.J., Bardwell, A.J. and Bardwell, L. (2001) Specificity of MAP Kinase Signaling in Yeast Differentiation Involves Transient versus Sustained MAPK Activation. *Mol Cell*, **8**, 683-691.

Sako, Y., Minoghchi, S. and Yanagida, T. (2000) Single-molecule imaging of EGFR signalling on the surface of living cells. *Nat Cell Biol*, **2**, 168-172.

Saouaf, S.J., Wolven, A., Resh, M.D. and Bolen, J.B. (1997) Palmitylation of Src family tyrosine kinases regulates functional interaction with a B cell substrate. *Biochem Biophys Res Commun*, **234**, 325-329.

Sato, K., Sato, A., Aoto, M. and Fukami, Y. (1995a) c-Src phosphorylates epidermal growth factor receptor on tyrosine 845. *Biochem Biophys Res Commun*, **215**, 1078-1087.

Sato, K., Sato, A., Aoto, M. and Fukami, Y. (1995b) Site-specific association of c-Src with epidermal growth factor receptor in A431 cells. *Biochem Biophys Res Commun*, **210**, 844-851.

Satokata, I., Ma, L., Ohshima, H., Bei, M., Woo, I., Nishizawa, K., Maeda, T., Takano, Y., Uchiyama, M., Heaney, S., Peters, H., Tang, Z., Maxson, R. and Maas, R. (2000) Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. *Nat Genet*, **24**, 391-395.

Schaller, M.D., Hildebrand, J.D., Shannon, J.D., Fox, J.W., Vines, R.R. and Parsons, J.T. (1994) Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2- dependent binding of pp60src. *Mol Cell Biol*, **14**, 1680-1688.

Schechter, A.L., Stern, D.F., Vaidyanathan, L., Decker, S.J., Drebin, J.A., Greene, M.I. and Weinberg, R.A. (1984) The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. *Nature*, **312**, 513-516.

Schlaepfer, D.D., Broome, M.A. and Hunter, T. (1997) Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. *Mol Cell Biol*, **17**, 1702-1713.

Schlaepfer, D.D., Hanks, S.K., Hunter, T. and van der Geer, P. (1994) Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature*, **372**, 786-791.

Schlaepfer, D.D. and Hunter, T. (1997) Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. *J Biol Chem*, **272**, 13189-13195.

Schlaepfer, D.D., Jones, K.C. and Hunter, T. (1998) Multiple Grb2-mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. *Mol Cell Biol*, **18**, 2571-2585.

Schomberg, D.W., Couse, J.F., Mukherjee, A., Lubahn, D.B., Sar, M., Mayo, K.E. and Korach, K.S. (1999) Targeted disruption of the estrogen receptor-alpha gene in

female mice: characterization of ovarian responses and phenotype in the adult. *Endocrinology*, **140**, 2733-2744.

Schwabe, J.W., Chapman, L., Finch, J.T. and Rhodes, D. (1993) The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. *Cell*, **75**, 567-578.

Schwartzberg, P.L., Xing, L., Hoffmann, O., Lowell, C.A., Garrett, L., Boyce, B.F. and Varmus, H.E. (1997) Rescue of osteoclast function by transgenic expression of kinase- deficient Src in src-/- mutant mice. *Genes Dev*, **11**, 2835-2844.

Sebastian, J., Richards, R.G., Walker, M.P., Wiesen, J.F., Werb, Z., Derynck, R., Hom, Y.K., Cunha, G.R. and DiAugustine, R.P. (1998) Activation and function of the epidermal growth factor receptor and erbB-2 during mammary gland morphogenesis. *Cell Growth Differ*, **9**, 777-785.

Sefton, B.M., Hunter, T., Beemon, K. and Eckhart, W. (1980) Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. *Cell*, **20**, 807-816.

Segatto, O., Lonardo, F., Wexler, D., Fazioli, F., Pierce, J.H., Bottaro, D.P., White, M.F. and Di Fiore, P.P. (1991) The juxtamembrane regions of the epidermal growth factor receptor and gp185erbB-2 determine the specificity of signal transduction. *Mol Cell Biol*, **11**, 3191-3202.

Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A. and Greene, G.L. (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell*, **95**, 927-937.

Sicheri, F. and Kuriyan, J. (1997) Structures of Src-family tyrosine kinases. *Curr Opin Struct Biol*, 7, 777-785.

Sicinski, P., Donaher, J.L., Parker, S.B., Li, T., Fazeli, A., Gardner, H., Haslam, S.Z., Bronson, R.T., Elledge, S.J. and Weinberg, R.A. (1995) Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell*, **82**, 621-630.

Siegel, P.M., Dankort, D.L., Hardy, W.R. and Muller, W.J. (1994) Novel activating mutations in the neu proto-oncogene involved in induction of mammary tumors. *Mol Cell Biol*, **14**, 7068-7077.

Siegel, P.M. and Muller, W.J. (1996) Mutations affecting conserved cysteine residues within the extracellular domain of Neu promote receptor dimerization and activation. *Proc Natl Acad Sci U S A*, **93**, 8878-8883.

Siegel, P.M., Ryan, E.D., Cardiff, R.D. and Muller, W.J. (1999) Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. *Embo J*, **18**, 2149-2164.

Simoncini, T., Hafezi-Moghadam, A., Brazil, D.P., Ley, K., Chin, W.W. and Liao, J.K. (2000) Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature*, **407**, 538-541.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A. and McGuire, W.L. (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*, **235**, 177-182.

Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A. and et al. (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science*, **244**, 707-712.

Smith, M.R., DeGudicibus, S.J. and Stacey, D.W. (1986) Requirement for c-ras proteins during viral oncogene transformation. *Nature*, **320**, 540-543.

Snyder, M.A. and Bishop, J.M. (1984) A mutation at the major phosphotyrosine in pp60v-src alters oncogenic potential. *Virology*, **136**, 375-386.

Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J. and et al. (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell*, **72**, 767-778.

Soriano, P., Montgomery, C., Geske, R. and Bradley, A. (1991) Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell*, **64**, 693-702.

Sourla, A., Luo, S., Labrie, C., Belanger, A. and Labrie, F. (1997) Morphological changes induced by 6-month treatment of intact and ovariectomized mice with tamoxifen and the pure antiestrogen EM-800. *Endocrinology*, **138**, 5605-5617.

Spivack, J.G., Erikson, R.L. and Maller, J.L. (1984) Microinjection of pp60v-src into Xenopus oocytes increases phosphorylation of ribosomal protein S6 and accelerates the rate of progesterone-induced meiotic maturation. *Mol Cell Biol*, **4**, 1631-1634.

Spivack, J.G. and Maller, J.L. (1985) Phosphorylation and protein synthetic events in Xenopus laevis oocytes microinjected with pp60v-src. *Mol Cell Biol*, **5**, 3629-3633.

Spivak-Kroizman, T., Rotin, D., Pinchasi, D., Ullrich, A., Schlessinger, J. and Lax, I. (1992) Heterodimerization of c-erbB2 with different epidermal growth factor receptor mutants elicits stimulatory or inhibitory responses. *J Biol Chem*, **267**, 8056-8063.

Stacey, D.W., Roudebush, M., Day, R., Mosser, S.D., Gibbs, J.B. and Feig, L.A. (1991) Dominant inhibitory Ras mutants demonstrate the requirement for Ras activity in the action of tyrosine kinase oncogenes. *Oncogene*, **6**, 2297-2304.

Stehelin, D., Guntaka, R.V., Varmus, H.E. and Bishop, J.M. (1976a) Purification of DNA complementary to nucleotide sequences required for neoplastic transformation of fibroblasts by avian sarcoma viruses. *J Mol Biol*, **101**, 349-365.

Stehelin, D., Varmus, H.E. and Bishop, J.M. (1975) Detection of nucleotide sequences associated with transformation by avian sarcoma viruses. *Bibl Haematol*, 539-541.

Stehelin, D., Varmus, H.E., Bishop, J.M. and Vogt, P.K. (1976b) DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature*, **260**, 170-173.

Stephens, R.M., Loeb, D.M., Copeland, T.D., Pawson, T., Greene, L.A. and Kaplan, D.R. (1994) Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses. *Neuron*, **12**, 691-705.

Sternberg, M.J. and Gullick, W.J. (1989) Neu receptor dimerization. *Nature*, **339**, 587.

Sternberg, M.J. and Gullick, W.J. (1990) A sequence motif in the transmembrane region of growth factor receptors with tyrosine kinase activity mediates dimerization. *Protein Eng*, **3**, 245-248.

Stokoe, D. and McCormick, F. (1997) Activation of c-Raf-1 by Ras and Src through different mechanisms: activation in vivo and in vitro. *Embo J*, **16**, 2384-2396.

Stover, D.R., Becker, M., Liebetanz, J. and Lydon, N.B. (1995) Src phosphorylation of the epidermal growth factor receptor at novel sites mediates receptor interaction with Src and P85 alpha. *J Biol Chem*, **270**, 15591-15597.

Sudol, M. (1998) From Src Homology domains to other signaling modules: proposal of the 'protein recognition code'. *Oncogene*, **17**, 1469-1474.

Superti-Furga, G. (1995) Regulation of the Src protein tyrosine kinase. *FEBS Lett*, **369**, 62-66.

Takeda, K., Noguchi, K., Shi, W., Tanaka, T., Matsumoto, M., Yoshida, N., Kishimoto, T. and Akira, S. (1997) Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci U S A*, **94**, 3801-3804.

Thomas, J.W., Ellis, B., Boerner, R.J., Knight, W.B., White, G.C., 2nd and Schaller, M.D. (1998) SH2- and SH3-mediated interactions between focal adhesion kinase and Src. *J Biol Chem*, **273**, 577-583.

Thomas, S.M. and Brugge, J.S. (1997) Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol*, **13**, 513-609.

Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R.C. and et al. (1995) Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science*, **269**, 230-234.

Tice, D.A., Biscardi, J.S., Nickles, A.L. and Parsons, S.J. (1999) Mechanism of biological synergy between cellular Src and epidermal growth factor receptor. *Proc Natl Acad Sci U S A*, **96**, 1415-1420.

Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E. and Chambon, P. (1989) The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell*, **59**, 477-487.

Traverse, S., Gomez, N., Paterson, H., Marshall, C. and Cohen, P. (1992) Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem J*, **288**, 351-355.

Twamley-Stein, G.M., Pepperkok, R., Ansorge, W. and Courtneidge, S.A. (1993) The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH 3T3 cells. *Proc Natl Acad Sci U S A*, **90**, 7696-7700.

Tzahar, E., Pinkas-Kramarski, R., Moyer, J.D., Klapper, L.N., Alroy, I., Levkowitz, G., Shelly, M., Henis, S., Eisenstein, M., Ratzkin, B.J., Sela, M., Andrews, G.C. and Yarden, Y. (1997) Bivalence of EGF-like ligands drives the ErbB signaling network. *Embo J*, **16**, 4938-4950.

Umesono, K. and Evans, R.M. (1989) Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell*, **57**, 1139-1146.

van der Geer, P., Hunter, T. and Lindberg, R.A. (1994) Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu Rev Cell Biol*, **10**, 251-337.

van Genderen, C., Okamura, R.M., Farinas, I., Quo, R.G., Parslow, T.G., Bruhn, L. and Grosschedl, R. (1994) Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes Dev*, **8**, 2691-2703.

Vanacker, J.M., Pettersson, K., Gustafsson, J.A. and Laudet, V. (1999) Transcriptional targets shared by estrogen receptor- related receptors (ERRs) and estrogen receptor (ER) alpha, but not by ERbeta. *Embo J*, **18**, 4270-4279.

Varmus, H.E., Bishop, J.M. and Vogt, P.K. (1973) Appearance of virus-specific DNA in mammalian cells following transformation by Rous sarcoma virus. *J Mol Biol*, **74**, 613-626.

von Willebrand, M., Williams, S., Saxena, M., Gilman, J., Tailor, P., Jascur, T., Amarante-Mendes, G.P., Green, D.R. and Mustelin, T. (1998) Modification of phosphatidylinositol 3-kinase SH2 domain binding properties by Abl- or Lck-mediated tyrosine phosphorylation at Tyr-688. *J Biol Chem*, **273**, 3994-4000.

Waksman, G., Shoelson, S.E., Pant, N., Cowburn, D. and Kuriyan, J. (1993) Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: crystal structures of the complexed and peptide-free forms. *Cell*, **72**, 779-790.

Walker, F., deBlaquiere, J. and Burgess, A.W. (1993) Translocation of pp60c-src from the plasma membrane to the cytosol after stimulation by platelet-derived growth factor. *J Biol Chem*, **268**, 19552-19558.

Walter, A.O., Peng, Z.Y. and Cartwright, C.A. (1999) The Shp-2 tyrosine phosphatase activates the Src tyrosine kinase by a non-enzymatic mechanism. *Oncogene*, **18**, 1911-1920.

Wang, Y.Z., Wharton, W., Garcia, R., Kraker, A., Jove, R. and Pledger, W.J. (2000) Activation of Stat3 preassembled with platelet-derived growth factor beta receptors requires Src kinase activity. *Oncogene*, **19**, 2075-2085.

Wasilenko, W.J., Payne, D.M., Fitzgerald, D.L. and Weber, M.J. (1991) Phosphorylation and activation of epidermal growth factor receptors in cells transformed by the src oncogene. *Mol Cell Biol*, **11**, 309-321.

Waterman, H., Alroy, I., Strano, S., Seger, R. and Yarden, Y. (1999) The C-terminus of the kinase-defective neuregulin receptor ErbB-3 confers mitogenic superiority and dictates endocytic routing [In Process Citation]. *Embo J*, **18**, 3348-3358.

Webster, N.J., Green, S., Jin, J.R. and Chambon, P. (1988) The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. *Cell*, **54**, 199-207.

Weiner, D.B., Liu, J., Cohen, J.A., Williams, W.V. and Greene, M.I. (1989) A point mutation in the neu oncogene mimics ligand induction of receptor aggregation. *Nature*, **339**, 230-231.

Weiss, A. and Schlessinger, J. (1998) Switching signals on or off by receptor dimerization. *Cell*, **94**, 277-280.

Wels, W., Beerli, R., Hellmann, P., Schmidt, M., Marte, B.M., Kornilova, E.S., Hekele, A., Mendelsohn, J., Groner, B. and Hynes, N.E. (1995) EGF receptor and p185erbB-2-specific single-chain antibody toxins differ in their cell-killing activity on tumor cells expressing both receptor proteins. *Int J Cancer*, **60**, 137-144.

White, R., Sjoberg, M., Kalkhoven, E. and Parker, M.G. (1997) Ligand-independent activation of the oestrogen receptor by mutation of a conserved tyrosine. *Embo J*, **16**, 1427-1435.

Wiebe, V.J., Osborne, C.K., Fuqua, S.A. and DeGregorio, M.W. (1993) Tamoxifen resistance in breast cancer. *Crit Rev Oncol Hematol*, **14**, 173-188.

Wiesen JF, Young P, Werb Z, Cunha GR. (1999) Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development.Development.Jan;126(2):335-44.

Wilson, L.K., Luttrell, D.K., Parsons, J.T. and Parsons, S.J. (1989) pp60c-src tyrosine kinase, myristylation, and modulatory domains are required for enhanced mitogenic responsiveness to epidermal growth factor seen in cells overexpressing c-src. *Mol Cell Biol*, **9**, 1536-1544.

Winstanley, J., Cooke, T., Murray, G.D., Platt-Higgins, A., George, W.D., Holt, S., Myskov, M., Spedding, A., Barraclough, B.R. and Rudland, P.S. (1991) The long term prognostic significance of c-erbB-2 in primary breast cancer. *Br J Cancer*, **63**, 447-450.

Wright, J.D., Reuter, C.W. and Weber, M.J. (1996) Identification of sites on epidermal growth factor receptors which are phosphorylated by pp60src in vitro. *Biochim Biophys Acta*, **1312**, 85-93.

Xie, W., Paterson, A.J., Chin, E., Nabell, L.M. and Kudlow, J.E. (1997) Targeted expression of a dominant negative epidermal growth factor receptor in the mammary gland of transgenic mice inhibits pubertal mammary duct development. *Mol Endocrinol*, **11**, 1766-1781.

Xing, L., Venegas, A.M., Chen, A., Garrett-Beal, L., Boyce, B.F., Varmus, H.E. and Schwartzberg, P.L. (2001) Genetic evidence for a role for Src family kinases in TNF family receptor signaling and cell survival. *Genes Dev*, **15**, 241-253.

Xu, Y.H., Richert, N., Ito, S., Merlino, G.T. and Pastan, I. (1984) Characterization of epidermal growth factor receptor gene expression in malignant and normal human cell lines. *Proc Natl Acad Sci U S A*, **81**, 7308-7312.

Yaciuk, P., Choi, J.K. and Shalloway, D. (1989) Mutation of amino acids in pp60c-src that are phosphorylated by protein kinases C and A. *Mol Cell Biol*, **9**, 2453-2463.

Ylikomi, T., Bocquel, M.T., Berry, M., Gronemeyer, H. and Chambon, P. (1992) Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. *Embo J*, **11**, 3681-3694.

Young, M.A., Gonfloni, S., Superti-Furga, G., Roux, B. and Kuriyan, J. (2001) Dynamic coupling between the SH2 and SH3 domains of c-Src and Hck underlies their inactivation by C-terminal tyrosine phosphorylation. *Cell*, **105**, 115-126.

Yu, H., Chen, J.K., Feng, S., Dalgarno, D.C., Brauer, A.W. and Schreiber, S.L. (1994) Structural basis for the binding of proline-rich peptides to SH3 domains. *Cell*, **76**, 933-945.

Yu, Q., Geng, Y. and Sicinski, P. (2001) Specific protection against breast cancers by cyclin D1 ablation. *Nature*, **411**, 1017-1021.

Zeps, N., Bentel, J.M., Papadimitriou, J.M., D'Antuono, M.F. and Dawkins, H.J. (1998) Estrogen receptor-negative epithelial cells in mouse mammary gland development and growth. *Differentiation*, **62**, 221-226.

Zhang, H.T., O'Rourke, D.M., Zhao, H., Murali, R., Mikami, Y., Davis, J.G., Greene, M.I. and Qian, X. (1998) Absence of autophosphorylation site Y882 in the p185neu oncogene product correlates with a reduction of transforming potential. *Oncogene*, **16**, 2835-2842.

Zhang, Q.X., Borg, A., Wolf, D.M., Oesterreich, S. and Fuqua, S.A. (1997) An estrogen receptor mutant with strong hormone-independent activity from a metastatic breast cancer. *Cancer Res*, **57**, 1244-1249.

Zheng, X.M., Resnick, R.J. and Shalloway, D. (2000) A phosphotyrosine displacement mechanism for activation of Src by PTPalpha. *Embo J*, **19**, 964-978.

Zhou S, C.K.r., Eck MJ, Harrison SC, Feldman RA, Mohammadi M, Schlessinger J, Hubbard SR, Smith DP, Eng C, et al. (1995) Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. *Nature*, **373**, 536-539.

Zwijsen, R.M., Buckle, R.S., Hijmans, E.M., Loomans, C.J. and Bernards, R. (1998) Ligand-independent recruitment of steroid receptor coactivators to estrogen receptor by cyclin D1. *Genes Dev*, **12**, 3488-3498.

Zwijsen, R.M., Wientjens, E., Klompmaker, R., van der Sman, J., Bernards, R. and Michalides, R.J. (1997) CDK-independent activation of estrogen receptor by cyclin D1. *Cell*, **88**, 405-415.