ROLE AND MECHANISM OF ACTION OF
TYROSINE KINASES IN
MAMMARY TUMORIGENESIS

By
CHANTALE T. GUY, M.SC.

A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree
Doctor of Philosophy

McMaster University
(c) Copyright by Chantale T. Guy, July 1994
ROLE OF TYROSINE KINASES IN MAMMARY TUMORIGENESIS
DOCTOR OF PHILOSOPHY (1994) McMaster University
(Health Sciences) Hamilton, Ontario, Canada

TITLE: Role and mechanism of action of tyrosine kinases in mammary tumorigenesis

AUTHOR: Chantale T. Guy, B.Sc. (Montreal University)
         M.Sc. (Montreal University)

SUPERVISOR: Professor W.J. Muller

NUMBER OF PAGES: xvi, 193
ABSTRACT

Overexpression and amplification of the neu proto-oncogene have been implicated in the development of aggressive human breast cancer. To investigate the effect of mammary gland-specific expression of the neu proto-oncogene, transgenic mice carrying the unactivated neu gene under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer were established. Overexpression of neu in the mammary tumors was associated with elevated Neu intrinsic tyrosine kinase activity and the stochastic development of focal mammary tumors which frequently metastasized. These observations provide the first direct evidence that expression of the proto-oncogenic form of neu results in a heritable development of metastatic mammary tumors.

Another potent tyrosine kinase activity that has been implicated in the genesis of murine mammary tumors is that associated with polyomavirus middle T antigen (PyV MTAg). Expression of MMTV/PyV middle T antigen in the mammary glands of transgenic mice resulted in the induction of multifocal mammary tumors which frequently metastasized to the lung. The potent transforming activity of PyV MTAg can, in part, be attributed to its ability to associate with an activate a number of c-Src family tyrosine kinases (c-Src, c-Yes, and Fyn). In order to assess the role of individual members of the c-Src family of tyrosine kinases in PyV MTAg induced mammary tumorigenesis, I have crossed the MMTV/PyV middle T fusion gene with mice bearing disrupted c-src or c-yes alleles. Mice expressing the PyV middle
T transgene in the absence of functional c-Src rarely developed metastatic mammary tumors. However, transgenic mice expressing the PyV MTAg in mammary epithelium lacking functional c-Yes developed multifocal mammary tumors with kinetics comparable to MMTV/PyV middle T strains possessing a functional c-Yes. These findings suggest that c-Src tyrosine kinase activity is required for PyV MTAg induced mammary tumorigenesis and also illustrate a in vivo genetic approach to dissect mitogenic signal transduction pathways.
ACKNOWLEDGEMENTS

I am thankful to my advisor William J. Muller for his help, encouragement and inspiration which were always present during my Ph.D. I am also grateful to him for pushing me during deadlines and last minute engagements. Not that I have learned from it, but it did prevent me to go through 4 years of trouble. Finally, I wish to thank Bill for introducing me to a much broader scientific world which I had never suspected the existence before, and make me realize that everything is possible if you really want it.

I am also obliged to John A. Hassell and Ludvik Prevec for their input and advice on my work during discussions and committee meetings.

I am especially grateful to Marc Webster, David Dankort, Senthil Muthuswamy and Peter Siegel for their friendship, support, and their inexhaustible ways of keeping a humorous and fun atmosphere in the laboratory.

I am also deeply indebted to my friends Monica Graham and Laura Hasting for their trust, devotion and true friendship ever present during and after my Ph.D.

Finally, and none the less, I wish to thank my husband and friend, Claude M. Trépanier, for its constant encouragement, faith, and unquestionable love which I hope will never end.

Merci!!!
CONTRIBUTION OF OTHERS

Some of the work described in my thesis has been generated with the help and collaboration of friends and collaborators which I wish to quote.

Figure 3.6, Expression of Neu protein and associated kinase activity in tumor and adjacent mammary epithelium, was accomplished by Marc A. Webster, a Ph.D. student in the laboratory.

Figure 3.7, Induction of mammary tumors correlates with elevated Neu expression and the induction of several phospho-tyrosine kinase containing proteins, was completed by Mike Schaller, post-doctoral fellow in the laboratory of Dr. Tom Parsons.

Figure 5.1A and 5.10, Activation of the c-Src family of tyrosine kinases in the PyV middle T induced mammary tumors; Polyomavirus middle T antigen-associated c-Src kinase activity in mammary tumors of mice lacking functional c-Yes, was performed by Senthil K. Muthuswamy, a Ph.D. student in the laboratory.
CHAPTER 1: INTRODUCTION

1.1. Cancer, a genetic disease
   1.1.1. Introduction
   1.1.2. Oncogenes
   1.1.3. Tumor suppressor genes

1.2. Oncogenes involved in human breast cancer

1.3. Transgenic mouse models of mammary tumorigenesis
   1.3.1. Introduction
   1.3.2. Multistep mammary tumorigenesis in transgenic mice
      i) Cytoplasmic and nuclear oncogenes
      ii) Growth factors
   1.3.3. The role of tyrosine kinases in mammary tumorigenesis
      i) Expression of Neu
1.3.4. The role of PyV MTAg in mammary tumorigenesis

i) Association of middle T antigen with cellular tyrosine kinases

ii) Association of middle T antigen with PI3'kinase and protein phosphatase 2A

iii) Transgenic mouse models of PyV middle T antigen transformation

1.4. Experimental rationale

CHAPTER 2: MATERIALS AND METHODS

2.1. Generation of transgenic mice

2.1.1. DNA constructions

2.1.2. Preparation of DNA for microinjection

2.1.3. Isolation of eggs for microinjection

2.1.4. Embryo transfer

2.1.5. Identification of transgenic animals

2.2. DNA analyses

2.2.1. Southern blot analysis

i) Transfer of DNA to membrane

ii) DNA probe

iii) Preparation of radiolabeled probe

iv) Hybridization
2.3. Detection of mutations using Reverse Transcriptase/ Polymerase Chain Reaction (RT/PCR).

2.3.1. RT/PCR ................................................................................. 38
2.3.2. Detection of mutations ....................................................... 39
2.3.3. DNA Sequencing ................................................................. 40

2.4. RNA analysis.

2.4.1. Extraction of RNA from tissues ........................................ 40
2.4.2. RNase protection assays ................................................... 41
   i) Construction of templates ...................................................... 41
   ii) Synthesis of radiolabeled probe ......................................... 42
   iii) Hybridization ................................................................. 43
2.4.3. Northern blot analysis ....................................................... 44

2.5. Protein analysis.

2.5.1. Antibodies ...................................................................... 44
2.5.2. Protein extract preparation .............................................. 45
2.5.3. Immunoblotting ............................................................... 45
2.5.4. Immunoprecipitation ....................................................... 46
2.5.5. In vitro kinase and enolase assays ..................................... 47
2.5.6. Zymographic analysis ...................................................... 48

2.6. Histopathology.

2.6.1. Pathology ....................................................................... 49
2.6.2. Whole-mounts ............................................................... 50
2.6.3. Tissue transplants .......................................................... 51
CHAPTER 3: EXPRESSION OF THE NEU PROTO-ONCOGENE IN THE MAMMARY EPITHELIUM OF TRANSGENIC MICE INDUCES METASTATIC DISEASE.

3.1. Introduction .............................................................................................................................. 52
3.2. Results ....................................................................................................................................... 53
    3.2.1. Expression of the transgene correlates with tumor development ........................................... 53
    3.2.2. Mammary gland-specific expression of neu is associated with the induction of metastatic disease ................................................................. 68
    3.2.3. Induction of mammary tumors by neu results in elevated Neu tyrosine kinase activity .......... 69
    3.2.4. Induction of mammary tumors by neu results in the appearance of several phosphotyrosine containing proteins ........................................ 72
    3.2.5. Detection of mutations within the MMTV/unactivated neu transgene during mammary tumorigenesis ............................................................. 75
3.3. Discussion ................................................................................................................................... 78

CHAPTER 4: INDUCTION OF MAMMARY TUMORS BY EXPRESSION OF POLYOMAVIRUS MIDDLE T ONCOGENE: A TRANSGENIC MOUSE MODEL FOR METASTATIC DISEASE.

4.1. Introduction .............................................................................................................................. 83
4.2. Results.

4.2.1. Generation of MMTV/PyV middle T antigen mice and tissue specificity of transgene expression.................................................85
4.2.2. The MMTV/PyV middle T antigen-induced mammary tumors possess associated tyrosine kinase activity..........................94
4.2.3. Expression of the PyV middle T antigen in the mammary epithelium results in the generation of multifocal metastatic mammary tumors...........................................................................99
4.2.4. PyV middle T antigen induces metastatic disease...............102
4.2.5. Elevated proteolytic activity in the MMTV/PyV middle T induced mammary tumors is associated with metastatic progression.................................................................................................................106

4.3. Discussion..................................................................................110

CHAPTER 5: ACTIVATION OF THE C-SRC TYROSINE KINASE IS REQUIRED FOR THE INDUCTION OF METASTATIC MAMMARY TUMORS IN TRANSGENIC MICE.

5.1. Introduction..................................................................................117

5.2. Results.

5.2.1. Expression of the PyV middle T oncogene in the mammary epithelium results in activation of c-Src and c-Yes tyrosine kinases..................................................................................................................118
5.2.2. A functional c-Src is required for the rapid induction of metastatic mammary tumors.................................122

5.2.3. Detection of PyV middle T associated tyrosine kinase activity in the mammary tissue of c-Src deficient mice..............130

5.2.4. c-Yes is dispensable for PyV middle T mediated mammary tumorigenesis.........................................................137

5.3. Discussion..................................................................................................................................................147

CHAPTER 6: CONCLUSION.................................................................................................................................153

CHAPTER 7: REFERENCES.................................................................................................................................162
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Chapter 1:</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1 Structures of the receptor and non-receptor families of protein tyrosine kinases</td>
<td>18</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 2:</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>No figures</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 3:</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 3.1. Structure of the MMTV/unactivated neu transgene</td>
<td>54</td>
</tr>
<tr>
<td>Figure 3.2. Tissue specificity of transgene expression</td>
<td>57</td>
</tr>
<tr>
<td>Figure 3.3. Histopathology of the MMTV/unactivated neu transgenic mice</td>
<td>61</td>
</tr>
<tr>
<td>Figure 3.4. Kinetics of tumor occurrence of the MMTV/unactivated neu transgenic mice</td>
<td>64</td>
</tr>
<tr>
<td>Figure 3.5. RNase protection analysis of expression of the transgene RNA in tumor and adjacent mammary epithelium</td>
<td>66</td>
</tr>
<tr>
<td>Figure 3.6. Expression of Neu protein and associated kinase activity in tumor and adjacent mammary epithelium</td>
<td>70</td>
</tr>
<tr>
<td>Figure 3.7. Induction of mammary tumors correlates with elevated Neu expression and the induction of several phospho-tyrosine containing proteins</td>
<td>73</td>
</tr>
</tbody>
</table>
Figure 3.8. Detection of somatic mutations within the MMTV/unactivated neu mammary tumors..............................76

Chapter 4:
Figure 4.1. Structure of the MMTV/PyV middle T antigen transgene........86
Figure 4.2. Tissue specificity of transgene expression in the MMTV/PyV middle T antigen transgenic mice.......................89
Figure 4.3. Western/immunoblot analyses of mammary tumor derived from the MMTV/PyV middle T antigen mice.....................95
Figure 4.4. Mammary tumors derived from the MMTV/PyV middle T antigen strains possess middle T associated tyrosine kinase activity.................................................................97
Figure 4.5. Histopathology of MMTV/PyV middle T transgenic mice........100
Figure 4.6. The expression of middle T oncogene results in metastatic mammary adenocarcinomas............................................104
Figure 4.7. Evidence of elevated proteolytic activity in the MMTV/PyV middle T induced mammary tumors.........................107

Chapter 5:
Figure 5.1. Activation of the Src family of tyrosine kinases in the PyV middle T induced mammary tumors..........................120
Figure 5.2. Interbreeding strategy between the MMTV/PyV middle T antigen transgenic mice (MT#634) and the c-src null mice......124
Figure 5.3.  c-Src is required for the induction of mammary tumors
in the MMTV/PyV middle T antigen transgenic mice.............126

Figure 5.4.  Histopathology of the MMTV/PyV middle T antigen
mice carrying disrupted c-src alleles.....................................129

Figure 5.5.  Detection of the c-Src and c-Yes in MMTV/PyV
middle T mammary tissues defective in c-Src function..........132

Figure 5.6.  Detection of PyV middle T associated c-Yes kinase activity
in mammary tissues defective in c-Src function.......................134

Figure 5.7.  Comparison between pattern of tyrosine phosphorylated
proteins in mammary tissue of MMTV/PyV middle T
mice defective in c-Src function.............................................138

Figure 5.8.  Interbreeding strategy between the MMTV/PyV middle T
transgenic mice (MT#634) and the c-yes null mice.................140

Figure 5.9.  Kinetics of tumor occurrence of the MMTV/PyV middle T
antigen in a wild-type, heterozygous and homozygous
c-Yes backgrounds..................................................................142

Figure 5.10.  Polyomavirus middle T antigen-associated c-Src kinase
activity in mammary tumors of mice lacking
functional c-Yes.........................................................................145

Chapter 6.7: No figures
## LIST OF TABLES

### Chapter 1:
Table 1.1. Comparison of MMTV/, Wap/, and Metallothionein/oncogene transgenic mice..................................................................................8

### Chapter 2:
No Tables

### Chapter 3:
Table 3.1. Transgene expression and onset of tumors formation in the MMTV/unactivated neu mice.................................................................59

### Chapter 4:
Table 4.1. Transgene expression and onset of tumors formation in the MMTV/PyV middle T mice.................................................................91

### Chapter 5, 6, 7:
No Tables
CHAPTER 1
INTRODUCTION

1.1. Cancer, a genetic disease

1.1.1. Introduction

Based on epidemiological studies, it has been long recognized that genetic factors may be responsible for the uncontrolled growth seen in breast cancer. For example, familial clusters of breast cancer have recently been demonstrated and may be attributable to a hereditary component (King, 1980; Lynch et al., 1984; Ponder, 1990). In addition, the detection of damaged chromosomes in cancer cells (Rowley, 1984; Yunis, 1986) and the apparent connection between susceptibility to cancer and impaired ability of these cells to repair damaged DNA (Lehmann, 1982; Hanawalt and Sarasin 1986) provide further evidence supporting this assertion.

1.1.2. Oncogenes

The discovery of oncogenes in the late 1970s strengthened the notion that cancer is a genetic disease. Scientists, through the study of transforming retroviruses, demonstrated that oncogenes are genes that are capable of inducing or maintaining cell transformation (Stehelin et al., 1976; Weiss et al., 1982; Varmus et al., 1984). In fact, the belief that deregulation of oncogene expression is responsible for the conversion of a normal cell to a cancer cell derives from observations made initially with RNA and DNA tumor viruses.
These studies revealed that oncogenic transformation of cells is mediated by particular viral genes and that many of these genes have cellular counterparts called proto-oncogenes. Indeed, modification of cellular oncogenes has also been incriminated in tumorigenesis (Cantley, 1991).

The potential for proto-oncogenes to participate in tumorigenesis arises because their protein products are engaged in the control of a variety of physiological processes associated with normal cell growth and differentiation (Weinberg, 1984; Bishop, 1987; Cantley, 1991). Indeed, almost all proto-oncogenes encode products involved in proliferative signal transduction pathways. For example, the secreted proteins or growth factors $sis$/PDGF, $int$-2, and TGF-α, may ultimately be oncogenic because they stimulate cell growth via autocrine loops (Sporh et al., 1981; Coffey et al., 1987; Aaronson, 1991). Some oncogenes encode mutant forms of growth factor receptors such as $erbB$, $fms$, $trk$, and $erbB2$. These receptor-like products are known to transform cells by delivering continuous ligand-independent mitogenic signals (Sherr et al., 1985; Bargmann et al., 1986b; Hunter, 1987). Oncogenes also encode growth factor transducer, such as GTP-binding proteins (e.g., the ras family) and non-receptor tyrosine kinases (e.g., the src family) (Grunicke, 1990; Wynford-Thomas, 1991). Finally, other oncogenes encode nuclear factors (e.g., transcription factors such as $jun$, $fos$; the myc families) which can be activated due to the loss of negative regulatory domains (Hunter, 1989; McCormick, 1989; Varmus, 1989; Cooper, 1990).

Transformation assays and analysis of tumor cell lines have uncovered several mechanisms by which proto-oncogenes undergo genetic changes that confer tumorigenicity. For example, activation of oncogenes can involve
point mutations, gene amplification, deletions, and chromosomal translocations (Bishop, 1987; Testa, 1990; Solomon et al., 1991). As a result of these modifications, these oncogenes are rendered constitutively active or are expressed at excessive levels, leading to uncontrolled cellular proliferation.

1.1.3. Tumor suppressor genes

Cellular transformation can also be facilitated by mutation or deletion of growth suppressor genes (i.e., tumor suppressor genes) (Horowitz et al., 1990; Mulligan et al., 1990).

Inactivation of tumor suppressor genes may play an important role in the induction of many tumor types, including breast carcinoma (Varley et al., 1989; Marshall, 1991). For example, it has been shown that functional inactivation of the retinoblastoma (Rb-1; chromosome 13q14) and p53 (chromosome 17p) suppressor genes are pivotal events in tumor formation or progression (Lee et al., 1988; Tang et al., 1988; Malkin et al., 1990; Srivastava et al., 1990). Additionally, families with an inherited deletion or mutation of Rb or p53 have increased risks of breast cancer and other cancers (Malkin et al., 1990; Weinberg et al., 1990).

Besides Rb and p53, other potential tumor suppressor genes have recently been associated with the induction of breast carcinoma. These include the nm23-H1 (Steeg et al., 1988; Liotta et al., 1991; Leone et al., 1991), and BRCA1 (Hall et al., 1990; Bishop et al., 1993) genes. Unfortunately, the specific role played by these genes in mammary tumorigenesis is not yet fully understood.
1.2. Oncogenes involved in human breast cancer.

Multiple genetic events are also thought to be required in the induction of human breast cancer. Numerous studies have looked at amplification (and subsequent overexpression), deletion, and mutation of several classes of proto-oncogenes implicated in the genesis and progression of human breast cancers. For example, studies have detected amplification and consequent overexpression of the putative growth factor receptor encoded by the neu gene (human gene: c-erbB-2 or HER/2) in a large proportion of human epithelial carcinomas and particularly in primary breast cancers (9-33%) (King et al., 1985; Yokota et al., 1986; DiFiore et al., 1987; Hudziak et al., 1987; Slamon et al., 1987; van de Vijver et al., 1987; Berger et al., 1988; Slamon et al., 1989). Moreover, neu amplification and overexpression have been shown to be inversely correlated with good prognosis and patient survival (Slamon et al., 1987; Slamon et al., 1989; Borg et al., 1990).

The loss of heterozygosity at the ras locus was also detected in a large percentage of breast tumor biopsies (27%) (Theillet et al., 1986; Cline et al., 1987). Similar to observations with neu gene defects, ras abnormalities have also been associated with poor prognostic parameters, such as advanced stage of the disease and shortened survival of the patient. Although activation of the ras oncogenes has not been frequently detected in human breast cancers (Kraus et al., 1984; Zarbl et al., 1985), ras does seem to play a crucial role in rodent mammary tumors induced by chemical carcinogens (Edwards et al., 1988; Strange et al., 1989; Aguilar-Cordova et al., 1991).
The involvement of c-myc in human breast cancer has also been described. Amplification of the c-myc oncogene was found in about one-third of primary breast cancer cells (Escot et al., 1986; Varley et al., 1987; van de Vijver et al., 1989) and breast carcinoma cell lines (Kozbor and Croce, 1984). Deregulated expression of the c-myc oncogene has been associated with the development of mammary cancers but has not been predictive of aggressive tumor behavior (Escot et al., 1986; Varley et al., 1987; Garcia et al., 1989; van de Vijver et al., 1989).

Finally, amplification of DNA markers within the chromosome 11q13 region (int-2, hst, bcl-1, PRAD1/cyclin D1, and ems-1) occurs in a variety of human malignant tumors (reviewed in Lammie and Peters, 1991) and is associated in breast cancer with an unfavorable clinical course of disease (Zhou et al., 1988; Lidereau et al., 1988; Tsuda et al., 1989; Borg et al., 1991; Schuuring et al., 1992). Interestingly, no one to date has been able to detect expression of the int-2 and hst proto-oncogenes in these patients. However, cyclin D1/PRAD1 and ems-1 are both expressed at high levels and probably represent the driving force of the amplification unit (Schuuring et al., 1993).

1.3. Transgenic mouse models of mammary tumorigenesis.

1.3.1. Introduction

Breast cancer is a prevalent and poorly understood disease in the human population. It is generally viewed as a complex, genetic, multistep process involving a series of independent events, each of which creates an
incremental phenotypic aberration. For example, the capabilities for extended proliferation, invasion of adjacent tissue, and distant metastasis might each be acquired independently by a cancer cell.

Although epigenetic factors (including hormonal status, age of the patient, frequency of pregnancies, and presence of hormone receptors on the tumor cells) undoubtedly play an important role in the development of malignant breast tumors, genetic damage remains the critical event responsible for tumor formation (reviewed in: Bishop, 1987; Seemayer and Cavenee, 1989; Callahan and Campbell, 1989).

In early attempts to identify mutations associated with human breast carcinomas, investigators used biological assays for tumorigenic mutations (Aaronson and Tronick, 1985) or cytogenetic analysis of primary breast tumors in culture (Trent, 1985). More recently, with advances in molecular biology, it has been possible to detect alteration in the structure and expression of a number of oncogenes in human breast cancer biopsies.

Although most of these experiments have provided a significant amount of information regarding the role of oncogenes in mammary tumorigenesis, they do not view malignant progression in its natural in vivo context. For example, in vitro studies do not take into account the effects of epigenetic factors such as hormonal conditions, surrounding tissue, extracellular matrix, and humoral factors on tumor development. Thus, directly correlating in vitro observations to in vivo consequences can be misleading. To overcome the intrinsic limitations of these studies, a number of laboratories have turned to the transgenic mouse as an experimental animal model system to assess the tissue-specific action of oncogenes in vivo.
To direct the expression of oncogenes to the mammary epithelium of transgenic mice, a variety of mammary specific promoter elements, including the Mouse Mammary Tumor Virus Long Terminal Repeat (MMTV/LTR) (Donehower et al., 1981) and the Whey acidic protein gene (Wap; Campbell et al., 1984) promoters have been fused to a variety of oncogenes (see Table 1.1). Although both these regulatory sequences are transcriptionally active in the mammary epithelium, their behavior differs in several aspects. Because the MMTV/LTR promoter contains transcriptional elements that are regulated by steroid hormones such as glucocorticoids and progesterone, it is activated much earlier in pregnancy. In addition, the MMTV/LTR is expressed in a wide range of tissues including salivary glands, male accessory glands, several secretory glands of the head, lung, and kidney, and sometimes in the lymphoid system (Pattengale et al., 1989; Muller, 1991; Cardiff and Muller, 1993). In contrast, the Wap gene encodes a milk protein and its expression is restricted to mammary epithelial cells during the late pregnancy and lactation phases. Thus, the expression of oncogenes under the transcriptional control of the Wap promoter is strictly confined and dependent on the hormonal stimuli governing pregnancy and lactation (Andres et al., 1987; Pittius et al., 1988; Schoenenberger et al., 1988; Andres et al., 1991). Because of the difference in the temporal pattern of expression of these elements, differences in the phenotypes exhibited by transgenic mice bearing an MMTV/oncogene and a Wap/oncogene may be influenced by the differentiation status of the cells.
Table 1.1. Comparison of MMTV/, Wap/, and Metallothionein/ oncogene transgenic mice.
Table 1.1. Comparison of MMTV, WAP, Metallothionein/oncogene transgenic mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>Promoter</th>
<th>Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-myc</td>
<td>MMTV</td>
<td>Stochastic mammary adenocarcinomas; occasional testicular, B-cell, T-cell and mast cell tumors.</td>
<td>Stewart et al., 1984 ; Leder et al., 1986</td>
</tr>
<tr>
<td>c-myc</td>
<td>WAP</td>
<td>Frequent stochastic mammary adenocarcinomas</td>
<td>Schoenenberger et al., 1988</td>
</tr>
<tr>
<td>v-Ha-ras</td>
<td>MMTV</td>
<td>Stochastic mammary adenocarcinomas; diffuse hyperplasia of the Harderian gland; adenocarcinomas of salivary gland; lymphomas</td>
<td>Sinn et al., 1987; Tremblay et al., 1989</td>
</tr>
<tr>
<td>N-ras</td>
<td>MMTV</td>
<td>Stochastic mammary gland adenocarcinomas; epididymal hyperplasia; lung tumors</td>
<td>Mangues et al., 1990</td>
</tr>
<tr>
<td>c-Ha-ras</td>
<td>WAP</td>
<td>Solitary mammary adenocarcinomas with low frequency and long latency</td>
<td>Andres et al., 1987; 1988</td>
</tr>
<tr>
<td>Activated Neu</td>
<td>MMTV</td>
<td>Polyclonal, synchronous breast adenocarcinomas; salivary gland hyperplasia; epididymal hyperplasia</td>
<td>Muller et al., 1988</td>
</tr>
<tr>
<td>Activated Neu</td>
<td>MMTV</td>
<td>Stochastic mammary adenocarcinomas; hyperplastic epididymus, seminal vesicle and salivary gland</td>
<td>Bouchard et al., 1989</td>
</tr>
<tr>
<td>c-erbB2/Neu</td>
<td>MMTV</td>
<td>Lymphomas; facial adenocarcinomas</td>
<td>Suda et al., 1990</td>
</tr>
<tr>
<td>Wnt-1</td>
<td>MMTV</td>
<td>Diffuse hyperplasia of mammary gland; solitary mammary adenocarcinomas; salivary gland tumors</td>
<td>Tsukamoto et al., 1988; Kwan et al., 1992</td>
</tr>
<tr>
<td>Int-2</td>
<td>MMTV</td>
<td>Diffuse hyperplasia of mammary gland; solitary mammary adenocarcinomas; and hyperplastic epididymis</td>
<td>Muller et al., 1990; Orniz et al., 1991</td>
</tr>
<tr>
<td>Int-3</td>
<td>MMTV</td>
<td>Mammary and salivary adenocarcinomas; hyperplasia of epididymis</td>
<td>Iwamoto et al., 1990</td>
</tr>
<tr>
<td>ret</td>
<td>MMTV</td>
<td>Mammary adenocarcinomas</td>
<td>Jhappan et al., 1992</td>
</tr>
<tr>
<td>TGF-α</td>
<td>MMTV</td>
<td>Mammary adenocarcinomas</td>
<td>Matsui et al., 1990; Halter et al. 1992</td>
</tr>
<tr>
<td>TGF-α</td>
<td>metallothionein</td>
<td>Solitary mammary adenocarcinomas; fibrosis of pancreas; multicellular hepathomases</td>
<td>Jhappan et al., 1990; Sandgren et al., 1990; Tomell et al., 1992</td>
</tr>
<tr>
<td>hGH</td>
<td>WAP</td>
<td>Precocious mammary gland development and mlk protein synthesis.</td>
<td>Bchli et al., 1991</td>
</tr>
<tr>
<td>SV40 lage T</td>
<td>MMTV</td>
<td>Lymphomas, lung and kidney adenocarcinomas; mammary gland adenocarcinomas</td>
<td>Chol et al., 1988</td>
</tr>
<tr>
<td>B1A and E1B</td>
<td>MMTV</td>
<td>Gastric tumors</td>
<td>Koike et al., 1989</td>
</tr>
</tbody>
</table>
1.3.2. Multistep mammary tumorigenesis in transgenic mice.

i) Cytoplasmic and nuclear oncogenes

The concept that multiple genetic events are required for the induction of tumorigenesis initially derives from studies involving the interaction between polyomavirus (PyV) middle T and PyV large T antigens (Rassoulzadegan et al., 1982). Neither PyV middle T nor PyV large T antigens were able to transform rat embryo fibroblasts alone. However, coexpression of both oncogenes in these cells fully induced a tumorigenic phenotype. These observations suggested that the concerted action of nuclear and cytoplasmic oncogenes were required to transform the cell. Further evidence for this model derives from observations made with the ras and myc oncogenes. Coexpression of ras and myc in primary rat embryo fibroblast resulted in oncogenic transformation of these cells (Land et al., 1983). In an analogous fashion, the ras oncogene collaborated with the adenovirus E1A oncogene to induce malignant transformation of baby rat kidney cells (Ruley et al., 1983). These initial observations suggested that each oncogene acted in a distinct and complementary way to transform cells. Together, these results argue that cytoplasmic oncogenes signal through nuclear oncogene pathways and that deregulation of both components acts synergistically to transform cells.

Under certain circumstances, however, primary cells can be transformed by the expression of a single oncogene. For example, transfection of activated ras into primary fibroblasts resulted in the emergence of a small
minority of tumorigenic oncogene-bearing transfectants (Spandidos and Wilkie, 1984; Land et al., 1986). Similar results were obtained when monolayers of embryo fibroblast were infected with Harvey sarcoma virus. When the virus was allowed to spread through the monolayer, thereby infecting the great majority of cells, fully transformed refractile cells appeared, and these were also tumorigenic (Land, 1986; Dotto et al., 1988). In both of these examples, transformation was achieved by the action of a single oncogene, conflicting with the model that requires at least two oncogenes for transformation.

One potential explanation for these discordant observations originates from the type of environment by which the transformants are surrounded during the respective experiments. Because ras was cointroduced with a neomycin resistance marker and used for selection, most of the transfectants expressed activated ras thus suppressing the inhibitory influence of neighboring normal cells (Bignami et al., 1988a). These observations strengthen the notion that the environment of an oncogene-bearing cells constitutes a strong determinant of their future growth properties.

In order to test this hypothesis in vivo, several oncogenes were introduced and expressed in a variety of tissues of transgenic mice (for a review, see Cory and Adams, 1988; Jaenisch, 1988; Hanahan, 1988). In particular, the transgenic mouse mammary model has largely contributed to the belief that, in addition to oncogene expression, full malignant transformation of the mammary epithelial cell requires additional genetic events. The first examples of transgenic strains behaving in this manner were the MMTV/c-myc (Stewart et al., 1984; Leder et al., 1986; Sinn et al., 1987)
and Wap/c-myc mice (Schoenenberger et al., 1988; Andres et al., 1988). These transgenic mice developed primarily solitary mammary tumors that appeared in a stochastic fashion after a long latency period (Andres et al., 1988; Schoenenberger et al., 1988). Because tumor formation did not coincide with the onset of c-myc expression, which was also detected in morphologically normal epithelium adjacent to the tumor, it was argued that c-myc is necessary but not sufficient to transform the mammary epithelium fully in vivo. These results argue that multiple genetic events are required for complete transformation to occur. Consistent with these observations, infection of primary mouse mammary epithelial cells with retroviruses bearing v-myc resulted in a preneoplastic pattern of ducts when implanted into cleared mammary fat pads (Edwards et al., 1988; Strange et al., 1989). Again, these results suggested that c-myc needed to collaborate with other genes in order to fully transform the mammary epithelium (Edwards et al., 1988; Strange et al., 1989).

One indication of the nature of these complementary events comes from a series of experiments in which v-Ha-ras was shown to complement c-myc in the transformation of primary rodent fibroblast in vitro (Land et al., 1983).

To test directly whether co-expression of v-Ha-ras with c-myc in the mammary epithelium is sufficient for mammary tumorigenesis, separate strains of transgenic mice expressing the MMTV/v-Ha-ras (Sinn et al., 1987; Tremblay et al., 1989) and Wap/ras (Andres et al., 1987; Andres et al., 1988; 1991) were generated and interbred with the respective c-myc transgenic strains. Like the c-myc transgenic mice, expression of the activated ras
oncogene also led to the stochastic formation of mammary tumors. Interestingly, other growth disturbances such as Harderian gland hyperplasia, salivary neoplasms, and lymphomas were also described in these models (Sinn et al., 1987; Tremblay et al., 1989; Mangues et al., 1990).

However, when both the MMTV/c-myc and MMTV/v-Ha-ras or Wap/c-myc and Wap/activated ras transgenic strains were crossed to produce dual carrier mice, these animals displayed a dramatic acceleration in the kinetics of tumor formation. Thus these two oncogenes can act in a synergistic fashion to transform the primary mammary epithelial cell (Sinn et al., 1987; Andres et al., 1988). Because these mammary tumors arose adjacent to morphologically normal epithelium, which also expressed the transgene, and because of their stochastic and clonal nature, it was concluded that even in the presence of these two oncogenes, further genetic events are required for complete malignant transformation of primary mammary epithelial cells (Sinn et al., 1987; Andres et al., 1988). Thus, in contrast to the tissue culture system, the nature of events required to establish the fully malignant phenotype in vivo are likely to be more complex than activation of a single oncogene.

ii) Growth factors

Besides cytoplasmic and nuclear oncogenes, other molecules such as the growth factors Wnt-1 and int-2 are believed to be important in mammary tumorigenesis (Nusse, 1988; Nusse et al., 1991). Both Wnt-1 and int-2 were initially implicated in mammary tumorigenesis because of the observation
that they were frequent targets for proviral integration during MMTV-mediated mammary carcinogenesis (Nusse, 1988). The putative role for Wnt-1 as a mammary oncogene was greatly strengthened by the demonstration that transgenic mice expressing this gene in the mammary epithelium developed mammary duct hyperplasia that progressed to malignancy (Tsukamoto et al., 1988; Kwan et al., 1992). However, the low incidence of tumor formation in these MMTV/Wnt-1 mice suggested that additional events were required for complete malignant transformation of the epithelium. The MMTV/int-2 transgene also induced pronounced mammary gland hyperplasia, but full malignancy developed infrequently and late in life (Muller et al., 1990; Kwan et al., 1992; Ornitz et al., 1992). As observed with other MMTV/oncogene strains, the stochastic occurrence of these mammary tumors argued that expression of either Wnt-1 or int-2 alone was not sufficient for mammary tumorigenesis to occur. To directly test whether the carcinogenic effects of int-2 can be detected more readily in the presence of an activated Wnt-1 gene, the MMTV/int-2 and MMTV/Wnt-1 transgenic mice were interbred. The bi-transgenic animals coexpressing Wnt-1 and int-2 provided the first direct evidence that a collaboration between two growth factors could transform the mammary epithelium. However, the phenotype observed in these animals also argued that coexpression of the growth factors was not sufficient for tumorigenesis (Kwan et al., 1992).

Another growth factor which has been implicated in the regulation of mammary gland development and tumorigenesis is the transforming growth factor-α (TGF-α). TGF-α shares sequence homology with the epidermal
growth factor (EGF), and binds to the same receptor. TGF-α is also known to play a role in the development of the mammary gland. For example, TGF-α is normally expressed in human and mouse mammary glands (Lisciа et al., 1990) and is capable of causing lobuloalveolar development of the gland in organ culture (Vonderharr, 1988). Subcutaneous implantation of pellets containing TGF-α has also been shown to stimulate epithelial proliferation in the mouse (Silberstein and Daniels, 1987). TGF-α is also able to act as a potent mitogen in a number of other epithelial cell systems (Carpenter and Cohen, 1979). The secretion of TGF-α by human mammary tumor cells suggests a potential role for TGF-α in malignant transformation. To test this hypothesis, transgenic mice carrying either an MMTV/TGF-α (Matsui et al., 1990; Halter et al., 1992) or a metallothionein/TGF-α (Jhappan et al., 1990; Sandgren et al., 1990) fusion gene have been generated. In both sets of animals, overexpression of TGF-α in the mammary epithelium was associated with extensive alveolar hyperplasia that predisposed mammary epithelium to neoplasia. Because of the wide spectrum of tissues in which genes driven by the metallothionein promoter are expressed, a variety of other growth disturbances were also detected in these transgenic strains. These disturbances included pancreatic metaplasia and hepatocellular carcinomas (Jhappan et al., 1990; Sandgren et al., 1990). Taken together, these observations argue that overexpression of TGF-α, while not sufficient for mammary tumorigenesis, predisposes the epithelium to subsequent events that are required for full malignant transformation.
More recently, the human growth hormone (hGH) was also investigated as a possible oncogenic factor in transgenic mice. The ubiquitously expressed hydroxymethyl-glutaryl coenzyme-A reductase (HMGCoA) promoter was used to overexpress the hGH gene. GH overexpression resulted in precocious mammary gland growth, development, and milk synthesis. Furthermore, post-lactation mammary gland regression did not occur and glandular differentiation persisted abnormally (Bchini et al., 1991). Transgenic animals overexpressing hGH also developed mammary tumors. Consistent with a potential role of hGH in mammary tumorigenesis, one strain of metallothionein/hGH mice also developed mammary carcinoma (Törnell et al., 1991; 1992).

1.3.3. The role of tyrosine kinases in mammary tumorigenesis

i) Expression of Neu

Although most of the transgenic models of mammary tumorigenesis seem to be consistent with the multi-step nature of cancer, several transgenic strains expressing activated tyrosine kinases develop multifocal mammary tumors within a very short period of time. The best characterized model of rapid tumor progression are transgenic mice bearing the activated rat neu gene under the transcriptional control of the MMTV/LTR. These animals invariably develop malignant transformation of the mammary epithelium that arises in a synchronous fashion (Muller et al., 1988).

Activated neu is a member of one of a large group of oncogenes that
encode protein tyrosine kinases (PTKs), which can be further subdivided into two families: the transmembrane receptor family (e.g., neu) and the cytosolic non-receptor family (e.g., c-src) (Hunter et al., 1991) (see Fig. 1.1). PTKs of the receptor family have a cytosolic domain that shares sequence homology with the kinase domain of the cytosolic nonreceptor members, but otherwise differ in structure. They have an extracellular ligand binding domain, a single transmembrane domain, and lack the SH-2 and SH-3 domains common to the nonreceptor tyrosine kinase family. The activity of the receptors is regulated by ligand binding to the extracellular domain (reviewed by Ullrich and Schlessinger, 1990). This extracellular domain may allow adjacent cytosolic domains within a receptor dimer to cross-phosphorylate each other on tyrosine residues, thereby causing a conformational change that enhances kinase activity for other substrates. It has also been shown that PTK activity is essential for mitogenic signaling by these receptors.

Oncogenic variants of the receptor family of PTKs also arise as a result of single amino acid substitutions in the transmembrane domain, or mutations in the cytosolic domain (reviewed by Ullrich and Schlessinger, 1990). These modifications elicit constitutive protein-tyrosine kinase activity in the absence of ligand and the creation of binding sites for recruitment of specific enzymes that transduce signals to the cell interior.

Several studies have demonstrated a correlation between the amplification and overexpression of the human growth factor receptor c-neu (c-erbB-2) and the induction of breast cancer (King et al., 1985; Yokota et al., 1986; Slamon et al., 1987; Gullick et al., 1991). In human breast tumors in which c-erbB-2 is overexpressed, no similar transforming mutation has yet
Figure 1.1. Structures of the receptor and non-receptor families of protein tyrosine kinases.
been identified (Kraus et al., 1987; Lemoine et al., 1990). However, overexpression of unmutated c-erbB-2 may lead to its constitutive activation (Di Marco et al., 1990). Given the close correlation between Neu overexpression and breast cancer, a number of laboratories have been interested in directly testing the tumorigenic potential of the neu oncogene in the mammary epithelium of transgenic mice. Several strains bearing MMTV/activated rat neu gene were generated, and early onset of transgene expression in the mammary epithelium was associated with the synchronous development of tumors involving the entire mammary epithelium (Muller et al., 1988). Moreover, in another set of experiments, direct injection of a retroviruses carrying the neu oncogene into the nipple of rat lead to the rapid formation of multifocal mammary carcinoma (Wang et al., 1991). In contrast to the multi-step process usually seen in other transgenic models, these results suggested that expression of activated neu requires few, if any, additional genetics events to transform the mammary epithelial cell (Muller et al., 1988). Expression of activated neu in other strains of transgenic mice gave rise to stochastic development of mammary tumors (Muller et al., 1988; Bouchard et al., 1989). The discrepancy in the phenotype exhibited by these mice may reflect differences in the level of transgene expression between the respective strains. Consistent with this hypothesis, the development of mammary tumors in MMTV/ret tyrosine kinase mice was closely correlated with the levels of transgene expression (Iwamoto et al., 1990).
1.3.4. The role of PyV middle T antigen in mammary tumorigenesis

Another potent tyrosine kinase activity that has been implicated in the genesis of murine mammary carcinogenesis is that associated with the polyomavirus (PyV) middle T antigen (MTAg). Infection of newborn mice with PyV resulted in the formation of a wide range of epithelial and mesenchymal tumor types, of which mammary adenocarcinomas represented a significant proportion (Reviewed by Dawe et al., 1987). Likewise, infection of adult female athymic nu/nu mice with PyV also resulted in the induction of mammary tumors with a high frequency of 96% (Berrebi et al., 1988; Haslam et al., 1992). Interestingly, induction of other tumor types was rare (Berrebi et al., 1988; Haslam et al., 1992). Taken together, these data suggest that polyoma-induced transformation of the mammary gland may involve an oncogene deregulation pathway similar to those involved in human breast tumors.

The tumorigenic potential of polyomavirus is localized to the early region of the PyV genome (Chowdhury et al., 1980; Hassell et al., 1980). The early region encodes three different proteins that are derived by alternative splicing of a single primary transcript (Treismann et al, 1981): (1) large T antigen (PyV LTAg), which is a 100 kDa nuclear protein required for viral replication; (2) middle T antigen (PyV MTAg), a 56 kDa membrane protein with transforming activity (Ito and Spurr, 1980; Treisman et al., 1981); and (3) small T antigen (PyV STAg), a 22 kDa protein that promotes growth in some assays (Noda et al., 1986).
The genetic analysis of cell transformation with polyomavirus has been significantly advanced by the generation of cDNA clones encoding the individual polyoma early gene products (large, middle, and small T antigen). Separate expression of these individual cDNAs allowed the uncoupling of transformation parameters and the designation of transformation functions to each of the respective products (Treismann et al., 1981; Rassoulzadegan et al., 1982; Cuzin et al., 1984; Mes and Hassell, 1982). Middle T antigen has been identified as the principle transforming oncoprotein of PyV since it can efficiently induce phenotypic transformation of established mouse and rat cell lines (Treismann et al., 1981; Rassoulzadegan et al., 1982; Magnusson et al., 1981). However, transfection of primary rat embryo fibroblasts with PyV MTAg cDNA failed to induce cellular transformation (Rassoulzadegan et al., 1983). Consistent with these results, injection of cDNA encoding middle T antigen also failed to induce tumor formation in rats (Asselin et al., 1983). Conversely, other studies have shown that expression of PyV middle T antigen could trigger the complete malignant transformation of primary cells (Spandidos and Riggio, 1986; Kaplan et al., 1985; Kornbluth et al., 1986). For example, a murine retrovirus encoding the PyV middle T antigen was able to fully transform non-established chicken embryo cells (Kaplan et al., 1985). In addition, when linked to strong transcriptional enhancer elements, the PyV MTAg could transform early passage rodent cells (Spandidos and Riggio, 1986). Therefore, under certain circumstances, the PyV MTAg does not require cooperating oncogenes to induce malignant conversion of specific cell types.
Middle T antigen is a phosphoprotein (Smith et al., 1979; Schaffhausen et al., 1979; Eckhart et al., 1979) of 421 amino acids that resides in cellular membranes. In polyoma virus infected cells, MTAg is found in two forms of 56 kDa and 58 kDa, depending on its state of phosphorylation (Schaffhausen et al., 1981). Biochemical fractionation studies and mutational analysis have demonstrated that most of the MTAg is anchored in the plasma membrane on the cytoplasmic side by virtue of a stretch of 22 hydrophobic amino acids at its carboxyl terminus. Furthermore, membrane localization is essential for MTAg to function in transformation since mutants defective in this property fail to transform cells (Schaffhausen et al., 1982; Markland et al., 1986; Templeton et al., 1984). However, the mechanism by which MTAg is inserted in the membrane remains uncertain.

i) Association of middle T antigen with cellular tyrosine kinases.

Biochemical analyses of PyV middle T antigen revealed that it is a phosphoprotein. Although most of the phosphorylation of MTAg occurs on serine and threonine residues, analysis of middle T immunoprecipitates from PyV transformed or infected cells revealed significant amounts of tyrosine phosphorylation (Eckhart et al., 1979; Schaffhausen et al., 1979; Schaffhausen et al., 1981).
Since MTAg has no known intrinsic kinase activity, its tyrosine phosphorylation is due to its association with and activation of cellular tyrosine kinases (Courtneidge et al., 1983; Bolen et al., 1984; Kornbluth et al., 1986; Courtneidge and Hebner, 1987; Kaplan et al., 1987; Cheng et al., 1988; Kypta et al., 1988; Pallas et al., 1988; Pallas et al., 1990; Walter et al., 1990). The importance of MTAg-associated tyrosine kinase activity in PyV transformation and oncogenesis is further supported by the finding that all transformation-competent strains of PyV possess this activity, while transformation-defective PyV strains generally lack detectable MTAg-associated tyrosine kinase activity (Carmichael et al., 1982; Bolen and Israel, 1985; Schaffhausen et al., 1981; Templeton and Eckhart, 1982).

There is compelling evidence indicating that MTAg specifically associates with and activates members of the c-Src family, including c-src itself (pp60) (Courtneidge and Smith, 1983; Bolen et al., 1984; Courtneidge et al., 1984), c-yes (pp62) (Kornbluth et al., 1987), and fyn (pp59) (Cheng et al., 1988; Kypta et al., 1988; Horak et al., 1989). In addition, polyomavirus mutants which encode MTAg that fail to associate with c-Src or c-Yes also fail to transform cells (Markland and Smith, 1987; Cook and Hassell, 1990). Conversely, MTAg-transformed rat fibroblast lines producing high levels of antisense c-src transcripts revert to an untransformed phenotype (Amini et al., 1986). Activation of the c-Src family is one of the key steps leading to transformation by MTAg. Complex formation between c-Src and MTAg results in activation of the specific tyrosine kinase activity of c-Src (Bolen et al., 1984; Courtneidge, 1985). The region implicated in binding involves the amino terminus of MTAg (Cook and Hassell, 1990) and the carboxyl terminus
of c-Src, proximal to Tyr 527 (Piwnica-Worms et al., 1990), and this tyrosine residue fails to be phosphorylated in the complex (Cartwright et al., 1986; Cheng et al., 1988). On the other hand, c-Src associated with MTAg is constitutively phosphorylated on tyrosine 416 (the major site of autoprophosphorylation) (Cartwright et al., 1986; Bolen et al., 1984) and results in enhanced kinase activity. Thus, MTAg appears to activate c-Src at least in part by preventing phosphorylation of Tyr 527, either by increasing the access of tyrosine 527 to a phosphatase or by decreasing access to a kinase. Together, these results suggest that association of c-Src with PyV MTAg is required for cellular transformation in these established cell lines. However, whether this is also true in other cell types is unclear.

The association of MTAg with c-Yes (but not with Fyn) results in an increase in c-Yes tyrosine kinase activity. Furthermore, it was also determined that there is only one molecule of MTAg within any one of these complexes. These observations suggest that in any given cell, polyomavirus transformation involves the simultaneous deregulation of separate pathways controlling cellular proliferation (Cheng et al., 1990).
ii) Association of Middle T antigen with PI3' kinase and PP2A

In addition to association with the Src family of tyrosine kinases, PyV MTAg is also known to interact with the 85 kDa subunit of the phosphatidylinositol 3'-kinase (PI3'kinase) (Courtneidge and Hebner, 1987; Kaplan et al., 1987; Pallas et al., 1988). Although the precise role of PI3'kinase in regulating cell proliferation is unknown, studies of PyV MTAg mutants (Courtneidge and Heber, 1987; Kaplan et al., 1986; Whitman et al., 1986) suggest that PI3'kinase is necessary for transformation by the MTAg/c-Src complex. Also, the association of PyV MTAg with the PI3'kinase is required for its transforming activity in vivo (Talmage et al., 1989).

More recently, stable complexes between PyV MTAg and the regulatory and catalytic subunits of protein phosphatase 2A (PP2A) have also been detected (Pallas et al., 1990; Walter et al., 1990). However, the role of these complexes in oncogenesis is unknown.

iii) Transgenic mouse models of PyV middle T transformation

Studies of oncogene action in transgenic mice have shown that both viral and cellular oncogene expression is correlated with tumor formation and that many oncogenes show a cell-type specificity in their action in vivo (Cory et al., 1988; Hanahan, 1986; 1988). The viral oncogenes of papovaviruses are particularly interesting in establishing a correlation between in vitro transformation and in vivo tumorigenesis. The transforming genes of both simian virus 40 (SV40) and polyomavirus have been extensively studied in cultured cells, and have been shown to be tumorigenic in mice (Hargis and Maikiel, 1979; Abramckuz et al., 1984; Tooze, 1981; Eddy, 1982). Surprisingly, a
number of papovavirus early region genes that show a broad spectrum of expression in cell culture have a restricted expression and tumor profile in transgenic mice. For example, expression of the SV40 early region is restricted to a few tissue sites and leads to predominantly choroid plexus tumors (Brinster et al., 1984; Palmiter et al., 1985). Likewise, the JC virus and BK virus early regions show restricted expression in transgenic mice, leading to tumors that recapitulate their tropism in humans (Small et al., 1986). Because the natural host of PyV is the mouse and infection of newborn mice with the virus results in tumor formation in a broad range of tissues, PyV has distinguished itself as being one of the best systems to use to dissect the oncogenic process in vivo.

Several transgenic models have been generated in order to look at the role of PyV in transformation in vivo. Transgenic mice carrying polyoma early region cDNAs linked to the polyomavirus early promoter sequences have been generated (Bautch et al., 1987). These transgenic mice develop multifocal tumors involving the vascular endothelium (hemangiomas).

The transforming activity of MTAg was also tested in another series of experiments in which MTAg was fused to the rat insulin II promoter. In these experiments, the expression of the middle T oncogene in pancreatic beta cells had no phenotypic consequence (Bautch, 1989).

Further evidence for a role of PyV MTAg as an oncogene in endothelial cells derives from observations made with chickens infected with an avian PyV MTAg retrovirus. Expression of MTAg in the endothelial cells of the chicken resulted in the wide spread induction of hemanginomas (Kornbluth et al., 1986). Indeed, mouse chimeras carrying cells infected with a
murine PyV MT retrovirus also develop multifocal endotheliomas which result in embryonic lethality (Williams et al., 1988). Other transgenic mice carrying the entire polyomavirus early region consistently develop similar vascular tumors (Wang and Blautch, 1991). In addition to the vascular tumors, transgenic mice carrying the entire PyV early region also develop lymphangiomas, osteosarcomas and fibrosarcomas (Wang and Bautch, 1991). In contrast to the tumors induced in virally infected chimeras, no epithelial derived tumors were observed in these transgenic models.

More recently, transgenic mice carrying a fusion gene comprised of the thymidine kinase promoter linked to MTAg coding sequences were established (Aguzzi et al., 1990). These animals expressed the transgene in the central and peripheral nervous system and developed neuroblastomas (Aguzzi et al., 1990). Transgenic mice expressing PyV MTAg under the control of the immunoglobulin heavy chain (IgE) enhancer/promoter have also been established (Rassoulzadegan et al., 1990). Surprisingly, the expression of MTAg in these mice led to the occurrence of carcinomas in various organs including the salivary, thyroid, and mammary glands, and the liver. The unexpected expression of MTAg in these organs likely reflects the influence of the integration site. Thus, in certain circumstances, expression of the middle T can induce carcinomas.
1.4. Experimental rationale

The molecular basis for the events responsible for conversion of a normal cell to a tumor cell remains a major challenge in understanding oncogenesis. It is becoming increasingly clear from these experimental and clinical observations that activation of tyrosine kinase associated activities such as Neu and PyV MTAg plays a central role in the induction of mammary tumors. The generation of transgenic mouse models which express these tyrosine kinases in the mammary gland would greatly enhance our understanding of the molecular basis for their potent transforming activity. To accomplish this, I have generated transgenic mice which express either the neu proto-oncogene or the PyV middle T antigen under the transcriptional control of the MMTV LTR.

To test directly the oncogenic potential of the unactivated Neu protein in the mammary epithelium, six strains of transgenic mice carrying a MMTV/unactivated neu fusion gene were generated. Mammary gland-specific expression of the unactivated neu product in five of these lines did not interfere with normal mammary gland growth and functional development. However in the best characterized MMTV/neu strain, N#202, 50% of the female carriers developed focal mammary tumors by six months of age. Because most mammary tumors arising in these strains expressed higher levels of neu specific RNA and protein than the adjacent normal epithelium, overexpression of unactivated neu product appears to result in the induction of mammary adenocarcinomas. These observations support the hypothesis that elevated expression of neu associated kinase activity in
the mammary epithelium is associated with the development of breast cancer.

Given the potential ability of PyV middle T antigen to signal cell proliferation through a number of signal transduction pathways, and the likely capacity of PyV MTAg to induce mammary adenocarcinomas, we assessed its oncogenic potential in the mammary gland of transgenic mice. To accomplish this, we directed the expression of the middle T antigen to the mammary epithelium using a MMTV/PyV middle T antigen fusion gene. Expression of middle T antigen in several independent transgenic strains resulted in the synchronous appearance of multifocal tumors involving the entire mammary glands. Thus, expression of the middle T oncogene appears to result in rapid tumor progression of the mammary epithelium. Interestingly, many of the MTAg transgenic mice developed multiple metastases in the lung. The multifocal nature of these mammary tumors and the high incidence of metastatic disease observed in these strains have important implications for understanding the molecular basis of tumor progression.

While it is clear that the interaction of PyV middle T antigen with cellular proteins, such as c-src and c-yes, plays an important role in tumorigenesis, the relative contribution of each of these protein complexes to transformation remains to be defined. To directly assess the role of c-src in PyV MTAg induced mammary tumorigenesis, we crossed transgenic mice carrying the MMTV/PyV middle T oncogene with mice carrying a disrupted c-src or c-yes gene (Soriano et al., 1991). By contrast to the rapid induction of mammary tumors observed in the parental MMTV/PyV middle T transgenic
strains, mammary gland-specific expression of the PyV middle T antigen in mice defective in c-Src function led only to the development of cystic hyperplasia of the mammary gland which rarely progressed to full malignancy. These observations indicate that a functional c-Src is required for PyV middle T induced mammary tumorigenesis and metastasis, and that the mammary epithelium is particularly sensitive to activation of the c-Src signal transduction pathway.
Chapter 2

MATERIALS AND METHODS

2.1. Generation of transgenic mice.

2.1.1. DNA constructions.

To derive the pMMTV/MT construct, the plasmid pmT165 (Cook and Hassell, 1990) bearing the PyV middle T cDNA (bounded by nucleotides 154 to 1560) (Treisman et al., 1981) was cleaved with HindIII and EcoRI and inserted into corresponding HindIII and EcoRI sites of the pA9 derived expression vector, pMMTV-SV40 (Huang et al., 1981). The latter construct was establish by first inserting the PstI-to BamHI fragment bearing the simian virus 40 (SV40) small t splicing and polyadenylation signal from CDM8 (Seed et al., 1987) into the corresponding sites in plasmid Bluescript KS (Stratagene). Then, the MMTV LTR containing SalI-to-HindIII fragment derived from plasmid pMMTVneuNT (Muller et al., 1988) was cloned into the corresponding sites of Bluescript KS.

The recombinant plasmid pMMTV/neuN was established by inserting the HindIII-EcoRI fragment encoding the unactivated neu rat cDNA and SV40 polyadenylation and splicing signals from pSV2neuN (Bargmann et al., 1986b) into a MMTV LTR vector. The MMTV LTR containing vector, pA9 (Huang et al., 1981), was modified by the insertion of a HindIII linker at the SmaI site and subsequently digested with EcoRI. This modified MMTV LTR
vector was ligated with the HindIII-EcoRI fragment containing the unactivated neu plus t... SV40 sequences to generate the plasmid pMMTV/neuN. The plasmid pASV was constructed by insertion of the SV40 splicing and polyadenylation sequences contained within a BamHI to BglII fragment derived from pSV2gpt (Muller et al., 1988) into the vector pGEM4.

2.1.2. Preparation of DNA for microinjection.

pMMTV/MT and pMMTV/neuN plasmid DNAs were amplified by chloramphenicol treatment (Clewell et al., 1972) in E. coli, and supercoiled molecules were isolated by lysozyme-SDS lysis, followed by 2 cesium chloride (CsCl) density gradients (Clewell and Helsinki, 1972). The resulting 5.5 kb MMTV/MT fragment to be microinjected was obtained by cleavage of pMMTV/MT DNA with 4 U (each) of SalI and SpeI per μg of DNA for 1 hour. Similarly, the plasmid pMMTV/neuN was digested with 4 U of SphiI and EcoRI. Each DNA were electrophoresed through a 1% agarose gel, the fragment to be injected cut out and the DNA was electroeluted in TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) buffer. The eluate was extracted once with butanol, 4 times with phenol-chloroform (1:1) and 2 times with chloroform alone. The purified DNA was ethanol-precipitated, and the pellet was washed once with 70% ethanol, dried, and resuspended in water. The concentration was then determined by UV absorption at 260 nm, and the DNA quality and concentration were confirmed by electrophoresis of an aliquot onto an agarose gel. The DNA stock was stored at -20 °C and diluted to a concentration of 5 μg/ml with a solution of TE (10 mM Tris and 0.1 mM EDTA) before microinjection.
2.1.3. Isolation of eggs for microinjection.

Prior to the microinjection, FVB/NHd female mice (Taconic Farm, Germantown, NY) were superovulated with two gonadotropins: pregnant mare's serum (PMS; Organon Inc., NJ; 5 IU/PBS, injected intraperitoneally, 3 days before microinjection) and human chorionic gonadotropin (hCG; Sigma; 5 IU/PBS, injected intraperitoneally 24 hrs before the eggs were harvested). Superovulated FVB/NHd female mice were mated the night before microinjection with FVB/NHd male mice (Taconic Farm, Germantown, NY). Eggs and cumulus were release from the uterine tubules with 45° angle forceps and placed in M2 culture medium (Quinn et al., 1982; 94.66 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl₂·H₂O, 1.19 mM KH₂PO₄, 1.19 mM MgSO₄·7H₂O, 4.15 mM NaHCO₃, 20.85 mM HEPES, 23.28 mM sodium lactate, 0.33 mM sodium pyruvate, 5.56 mM glucose, 4 g/l BSA, 0.06 g/l penicillin G, potassium salt, 0.05 g/l streptomycin sulfate, 0.01 g/l phenol red) containing hyaluronidase (Sigma) at a concentration of 300 µg/ml. After removing the cumulus with hyaluronidase, the eggs were washed twice in M2 medium (without hyaluronidase) and place on a depression slide overlaided with paraffin oil. The eggs were viewed under a Nikon Diaphot inverted microscope with Nomarski differential interference contrast optics. Approximately 0.5 to 1 pl of DNA solution (500-1000 copies) were microinjected into the male pronucleus.

2.1.4. Embryo transfers.

Following microinjection, viable one-cell mouse embryos (determined by gross morphology) were washed once in M2 medium and transferred to
the oviducts of pseudopregnant Swiss-Webster mice (Taconic, Germantown, NY). The pseudopregnant female were mated to vasectomized Swiss-Webster males (Taconic, Germantown, NY) the evening before the transfer. The surgery was accomplished under a stereoscope using avertin (Aldrich) as anesthetic (given intraperitoneally at a concentration of 0.15-0.17 ml/g of body weight).

2.1.5. Identification of transgenic animals.

To identify transgenic progeny, genomic DNA was extracted from a 1.5 cm tail sections of 3-4 week pups, as previously detailed by Sinn et al (1987). Briefly, the tails were digested in proteinase K buffer (10 mM Tris pH 8, 100 mM NaCl, 10 mM EDTA, 0.5% SDS and 0.2 mg/ml of proteinase K) overnight at 52 °C. On the next morning, the mixture was extracted twice with phenol:chloroform (1:1), once with chloroform and precipitated with 100% ethanol. The nucleic acid pellets were resuspended in 100 µl of TE at an approximative concentration of 1 µg/ml, and 15 µl of DNA solution was digested with 30 U of the respective endonuclease enzymes for 1 hr at 37 °C. For the MMTV/PyV middle T antigen and MMTV/neu mice, the genomic digestions were done using 30 U of BamHI per reaction. The src and yes tail DNAs were respectively cleaved with EcoRI and PstI. After electrophoresis through a 1.0% agarose gel, the DNA was transferred to Gene Screen filters (Dupond, Canada) (Southern, 1975) and analysed for the presence of PyV middle T antigen, unactivated neu , src or yes transgenes by hybridization with specific cDNA probe radiolabelled with [α-32P] dCTP by random priming (Feinberg and Vogelstein, 1983).
2.2. DNA Analysis.

2.2.1. Southern Blot Analysis.

i) Transfer of DNA to membrane.

After electrophoresis, the agarose gel was submerged successively for 40 min under gentle agitation at room temperature, in denaturing (0.4 N NaOH and 0.6 M NaCl) and neutralizing (0.5 M Tris pH 7.5 and 1.5 M NaCl) solutions. The gel was then equilibrated in 20X SSC (1.5 M NaCl, 0.15 M sodium citrate) for 15 min and placed on a piece of 3 mm Whatman paper supported by a glass plate, with its ends soaking in a solution of 20 X SSC. A piece of Gene Screen nylon membrane (Dupont), cut to the size of the gel and presoaked in 20 X SSC, was also layered on the gel. Three additional squares of whatman paper and a pile of hand paper were then placed on top of the membrane in order to promote the capillarity and transfer of the denatured DNA on the membrane (Southern, 1975). After 16 h, the papers were removed, the nylon membrane was rinsed in 2 X SSC and DNA was linked to the membrane by UV crosslinking using the auto crosslinking setting on Stratalinker 1800 (Stratagene).

ii) DNA Probe

The DNA template used to determine the genotype of the MMTV/PyV middle T antigen transgenic mice correspond to the EcoR1-HindIII fragment of cDNA contained in the plasmid pMMTV/MTE8. The rat neu probe used for the MMTV/unactivated neu lines was a 875 bp BamHI fragment of the cDNA originally derived from the plasmid pSV2neuN (Bargmann et al.,
1986b). The genomic digests and pedigree of the src and yes null mice were done using probes kindly provided by P. Soriano. The probe used in this analyses were a 350 bp SalI-BamHI fragment from the 5' end of p12 (Soriano et al., 1991) and a c-yes specific probe (Soriano, unpublished observation).

iii) Preparation of radiolabeled probe

200 ng of purified DNA template in a volume of 7 µl was denatured at 100 °C for 5 min, and quenched on ice for 3 min or more. The condensate was collected by a rapid centrifugation and 2 µl of 10X cocktail C (0.5 M Tris pH 8, 0.05 M MgCl₂, 0.0005 M β-mercaptoethanol, and 0.1 M of each of dGTP, dTTP, and dATP), 5 µl of 2 mg/ml synthetic random oligonucleotide primers, 5 µl of [α-³²P] dCTP (10 mCi/ml, 3000 μCi/mmol) and 10 units of Klenow fragment of E. coli DNA polymerase 1 (BRL) were added to the DNA. The reaction was allowed to proceed for 45 min at 37°C and was terminated by the addition of 230 µl of TE buffer. In order to determine the percentage of incorporation, 1 µl of the reaction was quantitated in a Beckman scintillation counter. The radiolabelled probe was used at 10⁶ counts per minute (cpm)/ml and boiled for 5 min at 100°C before use.

iv) Hybridization

The crosslinked membranes were soaked for 15 min at 60°C in a prehybridization solution (0.25 M Na₂HPO₄, 0.25 M H₃PO₄, 15% formamide, 7% SDS, 1% bovine serum albumin fraction V (BSA; Sigma Cat.#6003 ). The radiolabelled probe was then added and the hybridization was allowed to proceed overnight at 60°C. The following morning, the membranes were
washed once for 15 min at room temperature in 0.15 M sodium phosphate buffer and 1% SDS and twice for 30 min at 60°C in the same buffer. The membranes were then air dried and exposed against Kodak XAR-5 film for 24 h with an intensifying screen at -70°C.

2.3. Detection of mutations within the MMTV/unactivated *neu* tumors using RT/PCR.

2.3.1. Reverse Transcriptase/ polymerase Chain Reaction (RT/PCR)

Oligonucleotides #1306, #1307, #1309, and 1310 were synthesized on an Applied Biosystems oligonucleotide synthesizer at the MOBIX Main Central Facility.

The polymerase chain reaction was performed by a modification of the method described by Saiki et al. (1985). cDNA was synthesized from total RNA derived from various mammary tumors and adjacent normal tissues of the transgenic line N#202. A 10 μg of total RNA and 1 μg of reverse and forward (#1309, #1310) oligonucleotide primers were combined in a total volume of 9 μl of H₂O and heated to 90°C for 3 min. After being cooled to room temperature, a solution was made of 50 mM Tris pH 8.3, 6 mM MgCl₂, 40 mM KCl, 1 mM dithiothreitol (DTT), 2.5 mM each deoxynucleoside triphosphate, 1 U of RNAsin (Promega) per μl, and 0.5 U of reverse transcriptase per μl. After incubation at 37°C for 2h. The reaction mixture was used for the PCR reaction or stored at -20°C. The PCR reaction contained 10 mM Tris pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, each deoxynucleoside
triphasphate at 200 μM, each reverse and forward (#1309, #1310) oligonucleotide primers at 0.01 μg/μl, 0.1% Triton X-100, and 0.03 U of Taq polymerase (Promega) per μl in a total volume of 100 μl. A 2 μl volume of the cDNA mixture was added, and the reaction was overlaid with mineral oil. The conditions for PCR with oligonucleotides #1309 and #1310 were 94°C for 45 s, 45°C for 30 s, and 72°C for 3 s (35 cycles).

2.3.2. Detection of mutations

Oligonucleotide #1306, corresponding to the sequence: 5'-ACG CCC ACT ACA GTT GCA AT-3' of the wildtype transmembrane domain of neu and oligonucleotide #1307, corresponding to the sequence 5'-AGC CCC TCT ACA GTT GCA AT-3' of the mutant transmembrane domain of the activated neu gene (Bargmann et al., 1986b) were used for this experiment. 100 ng of each oligonucleotide was phosphorylated in 50 mM Tris pH 7.6, 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA with 50-100 μCi of [γ-32P] ATP (Amersham, 3000-7000 Ci/mmol), and 5 units of polynucleotide kinase (Boehringer Mannheim) for 40 min at 37°C. Unincorporated nucleotides were removed by chromatography over a G-25 Sephadex column. The specific activity of each nucleotide was about 5 x 10⁸ cpm/μg. 10 μg of each of the RT/PCR products were electrophoresed through a 1% agarose gel. The gels were denatured for 30 min in 0.5 M NaOH with 0.6 M NaCl, neutralized for 45 min in 1.5 M NaCl and 1 M Tris pH 7.4, and transfered on gene Screen as described above. After crosslinking, the membranes were incubated at 57°C for 2 h in a solution containing 2.5 X SSPE, 10 X Denhardt's, 500 μg/ml salmon sperm DNA, 0.1% SDS, and then were incubated for 3 h in
an identical solution with the addition of $6 \times 10^6$ cpm/ml labeled oligonucleotide at 57°C. Following hybridization, the membranes were washed twice with 6X SSC for 10 min at room temperature, once at 57°C for 30 min. The membranes were exposed against Kodak XAR film for 2-4 days with an intensifying screen at -70°C.

2.3.3. DNA Sequencing.

The DNA sequencing of RT/PCR products was performed according to the protocol of United States Biochemical using Sequenase version 2.0. The M13 T7 and T3 primers were used to sequence the denatured DNA and 0.5 μl of 10 μCi/μl of $[\alpha-^{35}S]$ dATP was included in the reactions. The samples were run on a 6% urea/acylamide gel, at 1900 volts for 6 to 8 hours, dried and exposed for 36 h against Kodak XAR-5 film.

2.4. RNA Analysis.

2.4.1. Extraction of RNA from tissues.

RNA was isolated from tissues by the procedure of Chirgwin et al. (1979), using a CsCl sedimentation gradient modification. The tissues were dissected from the animal and homogeneized with a polytron (Texmar Company, Ohio, USA) in 4 ml of guanidine isothiocyanate (GIT, BRL) solution (4 M GIT, 25 mM sodium citrate and 0.1 M β-mercaptoethanol) for 30 sec. The mixture was then layered onto 4 ml of 5.7 M CsCl and 25 mM sodium acetate pH 5, and the RNA was pelleted by centrifugation using a
SW41.Ti rotor at 32,000 rpm for 18 hours. The GIT and CsCl layers were removed by aspiration using a pasteur pipette and the RNA pellet was resuspended in 500 µl of ice cold sterile water. The RNA was then precipitated with two volume of cold ethanol, pelleted by centrifugation at 12,000 g for 30 min at 4°C and resuspended in 50 µl of sterile water. The RNA yield was determined by UV absorption at 260 nm (1 OD_{260} = 40 µg/ml RNA) and samples were stored at -70°C for future use.

2.4.2. RNase protection assays.

i) DNA template constructions

DNA template for the Polyomavirus middle T riboprobe was obtained from the plasmid pSP65mT(HA) and contains a 203-bp HindIII-to-AccI fragment of the PyV early region (PyV sequences from nucleotides 165 to 368) (Soeda et al., 1980; Cook and Hassell, 1990) inserted into the HindIII and AccI sites of pSP65 (Promega). The neu-specific riboprobe corresponds to the SV40 component of the transgene (pASV) and protects a 784-nucleotide fragment. The β-casein riboprotection probe (Yoshimura et al., 1986) was cloned as a 205-bp PstI fragment in pSP64 vector (Promega) and encode the 5' half of the mRNA in the correct orientation to generate an antisense probe. Finally, the internal control plasmid rpl32 [27.3.7] (Dudov and Perry, 1984) encodes an XhoII-to-DraI fragment of the mouse ribosomal gene L32 inserted into the corresponding sites of plasmid Bluescript KS (Stratagene).
ii) Synthesis of radiolabelled probe.

In order to transcribe DNA template into complementary sequences found in mRNAs, pSP65mT(HA) was digested with HindIII, pASV with BamHI, 5' β-casein with PstI and rpL32(27.3.7) with XbaI. Once linearized, 1 µg of each template was transcribed with 30 units of the corresponding RNA polymerase (pSP65mT(HA)/SP6; pASV/T7, β-casein/SP6; rpL32/T3) for 45 min at 37°C in 25 µl of transcription cocktail (200 mM Tris pH 7.5, 30 mM MgCl₂, 20 mM Spermidine; 15 mM DDT, 40 units of RNase inhibitor RNasin [Boehringer Mannheim], 100 µCi [α-³²P] UTP [10 mCi/ml, 3000 MCI/mmol] 1 mM of each of rCTP, rATP, rGTP and 0.1 mM of rUTP. The reaction was spiked with an additional 30 units of RNA polymerase and incubated at 37°C for an other 30 min. Transcription was terminated by the addition of 20 units of RNase-free DNase I, 1 µl of 0.5 M of MgCl₂ and 20 µl of water and incubated for 10 min at 37°C. After bringing the volume of reaction up to 100 µl with water, one phenol-chloroform (1:1) extraction was performed and the transcription products were precipitated by the addition of 20 µg of RNase-free yeast tRNA (MRE 600, Boehringer Mannheim), 0.1 volume of sodium acetate and two volume of 100% ethanol. The riboprobes were then cooled at -70°C for 20 min and spun at 12,000 g in a microfuge for 30 min at 4°C. The resulting pellet was resuspended in 100 µl of sterile water. The extent of incorporation of ³²P into RNA transcripts was measured by spotting 1 µl of the riboprobe on duplicate Whatman-DE81 ion exchange filters. One filter was directly quantitated in a Beckmann scintillation counter to measure total cpm, while the other was washed once with 0.5 M of sodium phosphate buffer (250 mM Na₂HPO₄; 250 mM NaH₂PO₄·H₂O), rinsed with water and
dried with 100% ethanol to remove unincorporated [α-32P]UTP. Remaining radioactivity was then quantitated and percent of incorporation established.

iii) Hybridization.

The RNase protection assays were performed as described by Melton et al. (1984), using 10 µg of total cellular RNA and 5 x 10^5 cpm of riboprobe (prepared as mentioned above). The RNA and probe were incubated at 85°C for 10 min in the hybridization buffer (80% formamide, 40 mM piperazine-N, N'-bis (2-ethanesulfonic acid) (PIPES) pH 6.4, 1 mM EDTA pH 8.0, and 0.4 M NaCl) in order to denature secondary structure of the RNAs. The reactions were then quickly transferred to a water bath set at the annealing temperature of 50°C and the hybridization was allowed to proceed overnight. The following day, hybridization mixtures were cooled on ice and 300 µl of RNase digestion buffer (300 mM NaCl, 10 mM Tris pH 7.4, 5 mM EDTA pH 7.5, 2 µg/ml RNAase T1 and 40 µg/ml RNAase A) were added to each sample for 30 min at 37°C. RNA digestion was terminated by the addition of 0.5% SDS and 150 µg/ml of proteinase K (Boehringer Mannheim) and incubated for an additional 30 min at 37°C. The reactions were extracted once with an equal volume of phenol:chloroform (1:1) and subsequently precipitated with 20 µg of yeast tRNA and 2 volumes of ethanol. The RNA was recovered by centrifugation at 12,000 g in a microfuge for 30 min at 4°C, and the precipitate was resuspended in 10 µl of formamide loading buffer (80% formamide, 10 mM EDTA pH 8.0, 1 mg/ml xylene cyanol FF and 1 mg/ml bromophenol blue). The nucleic acids were denatured at 95°C for 5 min and resolved on a 6% urea acrylamide gel (40% acrylamide: 2% N,N'-methylene-bis-acrylamide,
7 M urea (BRL), 0.001% ammonium persulfate and 0.0005% N,N,N',N'-tetramethyl-ethylenediamine (TEMED) ) and ran at 1500 volts in TBE buffer (0.1 M Tris, 0.08 M Boric acid, 0.002 M EDTA pH 8). The gel was dried and exposed at -70°C against Kodak XAR-5 film in presence of intensifying screens.

2.4.3. Northern blot analysis.

Northern blot analysis was performed as described by Maniatis et al. (1989). 10 µg of total RNA were electrophoresed through 1% agarose-formaldehyde-N-morpholinopropanesulfonic acid (MOPS) at 50 V for 6 h. RNA was then transferred to nitrocellulose filter as described in section 2.2.1.i and UV crosslinked using the auto crosslinking setting on Stratalinker 1800 (Stratagene). The conditions for prehybridization, hybridization, and washing of RNA immobilized on filters are essentially the same as those used for DNA (refer to section 2.2.1.i).

2.5. Protein Analysis.

2.5.1. Antibodies.

The antibodies used to immunoprecipitate PyV middle T antigen were (1) rat monoclonal antibody PAb 815 provided by Joseph Bolen, (2) a polyclonal anti-serum prepared by M. Naujokas from tumor-bearing Norwegian rats injected with Py transformed cells expressing all three T antigens and (3) the Glu-Glu antibody (Grussenmeyer et al., 1985). Neu was
detected with a mouse monoclonal antibody 7.16.4 (Drebin, J.A., 1985). The c-Src protein was detected using the monoclonal antibody 327 (Mab327; Lipsich et al. 1983; Oncogene Sci.). A rabbit antipeptide polyserum, pyes6 (Sodol et al., 1986) provided by Sara Courtneidge, as well as a monoclonal antibody 3H9 were used to specifically detect c-yes protein (Sukewaga et al., 1990). A rabbit antipeptide polyserum, pfyn2 (Kypka et al., 1988), specific for fyn protein was also kindly provided by Sara Courtneige.

2.5.2. Protein extract preparation.

Tissue samples were frozen in liquid nitrogen and ground to a fine powder by using cooled pestle and mortar. Cells were rapidly lysed on ice for 30 min in TNE lysis buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 2.5 mM EDTA) containing 1 mM sodium orthovanadate, 10 mM sodium fluoride, 2 μg/ml aprotinin, and 10 mM leupeptin. After lysis, cellular debris were removed by centrifugation in a microfuge at 12,000 g for 20 min at 4°C. The supernatants were transferred to clean tubes and protein concentrations were measured using the Bradford Bio Rad assay kit (Bradford et al. 1976). Samples were used immediately or kept at -70°C for a short period of time.

2.5.3. Immunoblotting.

The protein concentrations were adjusted to 50 μg and diluted in TNE lysis buffer to obtain a final volume of 50 μl. 40 μl of 2X sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue) were added to the protein extract and sample were denatured for 10 min at 95°C. Proteins were resolved on a 9% SDS-
acrylamide gel, run at 65 volts overnight according to the method of Laemmli (1970). After electrophoresis, the proteins were transferred electrophoretically (45 volts for 5 hours at 4°C) to a polyvinylidine difluoride (PVDF) filter (Immobilon-P, Millipore). Following transfer, membranes were rinsed with PBS and blocked in PBS containing 5% of Carnation powdered skim milk (fatty acid free) for 2 hours. After washing three times (10 min each) with PBS/0.01% Tween-20, the membranes were incubated for 2 hours at room temperature with anti-middle T (PAb 815), anti-neu, anti-c-src (327) or anti-cyes (pyes6) antibodies diluted 1:100 in PBS/5% skim milk. The membranes were washed three more times and incubated for 30 min at room temperature under agitation, with the appropriate secondary antibody conjugated to horse radish peroxidase (HRP, Promega) diluted 1:5000 in PBS/0.03% Tween-20. Finally, the membranes were washed four times as described above, rinse twice with PBS and proteins were revealed using the enhanced chemiluminescence (ECL, Amersham) detection system.

2.5.4. Immunoprecipitation.

In order to assayed for PyV middle T associated protein, a concentration of 500 μg of protein extract derived from mouse tissues (see above) was immunoprecipitated using 0.5 μl of specific polyclonal rat tumor serum for 2 hours at 4°C. The immune complexes were collected by adding 50 μl of a 20% solution of protein G-Sepharose beads in TNE lysis buffer for an additional 30 min. The beads were washed four times with TNE lysis buffer, once with PBS and resuspended in 80 μl of 2X sample buffer. All samples were denatured at 95°C for 10 min, then divided into two equal portions, resolved on duplicate
9% SDS-acrylamide gels and transferred into PVDF membranes. One half of each sample was assayed by immunoblotting for the presence in the immune complex of Src using Mab327 or Yes using pyes6 antibody and the other half was used to control for the level of middle T antigen in the complex using monoclonal antibody 815.

2.5.5. *In vitro* kinase and enolase assay.

*In vitro* kinase assays were conducted as described by Aguzzi et. al. (1990). The immunoprecipitation of PyV middle T antigen from mouse tissue extract was performed as described above. Neu was immunoprecipitated using 5 μl of the mouse monoclonal antibody 7.16.4. Src and Yes products were immunoprecipitated using respectively 5 μl of Mab327 or 5 μl of pyes6 antibody. After immunoprecipitation, *in vitro* kinase assays were performed by resuspending the beads with 25 μl of kinase buffer containing 20mM morpholinepropanesulfonic acid (MOPS) pH 7.0, 5 mM MgCl₂ and 5 μCi of [γ-³²P] ATP (10 mCi/ml, 3000 mCi/mmol) at 30°C for 20 min. The assay was terminated by washing the beads twice with TNE lysis buffer and adding 50 μl of 2X sample buffer. Enolase assays were also performed. Tissue extracts were incubated with an excess of antibodies specific to c-Src (Mab327; Oncogene Science), c-Yes (Mab3H9; Sukewaga et al., 1990), Fyn (pfyn2; Kypta et al., 1990) and PyV middle T antigen (Glu-Glu; Grussenmeyer et al., 1985) and incubated with 30 μl of protein G/Sepharose fast flow (Pharmacia) for 1 hr. Immunoprecipitates were washed five times with TNE buffer and once with 2X kinase buffer containing 200 mM HEPES at pH 7.0 and 10 mM MnCl₂. The beads were resuspended in 9 μl of 2X kinase buffer and a mixture containing
10μCi of [γ-32P]ATP (10 mCi/ml, 3000 mCi/mmol), and 10 μg of acid denatured enolase was added. The reaction mixture was incubated at room temperature for 5 min and stopped with sample buffer. The immunoprecipitated proteins were electrophoresed through 9% SDS-polyacrylamide gels, then fixed in 30% methanol-7% acetic acid for 20 min and finally soaked in 1 N potassium hydroxide at 55°C for 1 hour with gentle shaking. The base was neutralized with an equal volume of 1 N hydrochloric acid for 15 min, and rinsed with 30% of methanol before drying. Finaly, kinase activities were visualised after exposing dried gels to XAR-5 film for 1-12 hours with intensifying screens.

2.5.6. Zymographic analysis.

Substrate gels were used to localize enzyme activity by molecular weight. These gels differ from SDS-Laemmli gels in two aspects: the gels are made by incorporating the protein substrate of interest within the polymerized acrylamide matrix, and the samples are mixed with a higher concentration of SDS (without reducing agents) and not boiled. In my analysis, 1 mg/ml of gelatin Type I was used for substrate. 10% acrylamide gels were used to run the samples. Separating gels were prepared as follow: 7.5 ml of buffer A (1.5 M Tris-HCl pH 8.8, 0.4% SDS), 7.5 ml of 3 mg/ml of gelatin Type I in water, 10 ml of 30% acrylamide stock, 5 ml of water, 100 μl of 10% APS, and 15 μl of TEMED. The separating gels were allowed to set for 3 h at room temperature. Stacking gels were prepared as follow: 1.5 ml of 30% acrylamide, 2.5 ml of buffer B (0.5 M Tris-HCl pH 6.8, 0.4% SDS), 6 ml of water, 100 μl 10% APS and 15 μl TEMED. The protein samples (~100 μg) are
mixed 3:1 with 4X sample buffer (10% SDS, 4% sucrose, 0.25 M Tris pH 6.8, 0.1% bromophenol blue) and loaded on the substrate gels. The gels were run at 4°C to reduce enzyme interaction with the substrate during the run in 0.025 Tris, 0.19 M glycine, 0.1% SDS. Gels were run at 15 milli amps while in the stacking gel and 20 milli amps during the resolving phase. In order to monitor the progress of the electrophoresis, prestained molecular weight markers were used during the run. Following electrophoresis, the gel was soaked in 2.5% Triton-X-100 with gentle shaking for 30 min at room temperature with one change (this allows the proteins to renature by removal of SDS). The gel is then rinsed in substrate buffer (50 mM Tris-HCl pH 8, 5 mM CaCl$_2$) and incubated overnight in substrate buffer at 37°C under gentle shaking. At the end of the incubation, the gel is stained with Coomassie blue R 250 (Bio Rad) for 30 min with shaking and destained in water until clear bands are visible.

2.6. Histopathology

2.6.1. Pathology.

Development of tumors was detected by twice-weekly gross inspection of transgenic mice. The mice were sacrificed by cervical dislocation or CO$_2$ asphyxiation. Mice without tumors were killed at approximately 1 year of age.

Upon autopsy, tumors and tissues were fixed in 4% paraformaldehyde in PBS for 4 h, after which they were placed in 70% ethanol until
dehydration. The embedding, sectioning (5 micron), and staining (with Harris's hematoxylin and eosin) of tissues were performed by the Department of Pathology at McMaster University. Sections were examined for all grossly detected tumors and the diagnosis was confirmed by an expert pathologist, Dr. R. Cardiff.

2.6.2. Whole Mount.

The mice were killed by cervical dislocation or CO₂ asphyxiation and pinned to a cork bord ventral side up by placing pins through the tail and upper jaw. The skin was washed with ethanol and a midline incision was performed through the skin from external genitalia papillae at the base of the tail to the top of the lower jaw. More incisions were performed on both sides of external genitalia papillae through inguinal region to the base of the hind paws and from the jaw to the front paws. The mouse was skined by gently pulling the body wall away from the skin. The mammary fat pads were then exposed and dissected from the inner surface of the skin retaining some of the peripheral connective tissue. The glands were then stretched on glass slides and allowed to attach by brief air drying, preferably overnight. Glands were fixed and defatted overnight in acetone. To enhance the defatting process, the glands were squashed between two slides and returned to the acetone for an additional hour before staining. The glands were stained in Harris' hematoxylin overnight and destained with several changes of a solution of 2% HCl in ethanol. Destaining was considered complete when the epithelial component was seen in sharp contrast to the light background of the fat pad. After a brief (2-5 min) wash in 0.2% ammonium hydroxide, the slides were
transferred to 70% ethanol for several hours, followed by transfers to 95% and 100% ethanol for several hours. Finally, the tissue was cleared by an overnight exposure in toluene and were mounted in Permount and covered with coverslip.

2.6.3. Tissue transplants.

Pieces of mammary tumors or lung metastases, 3 to 5 mm³ in size, were removed from transgenic carriers and washed twice with 1 to 2 ml of Dulbecco modified Eagle medium. Tumor samples were transplanted into an anaesthetized syngeneic animal through an incision in the mammary fat pad. Recipient mice were examined twice weekly to determine tumor latency (time from transplant until tumors were first palpable) and tumor size.
CHAPTER 3

EXPRESSION OF THE NEU PROTO-ONCOGENE IN THE
MAMMARY EPITHELIUM OF TRANSGENIC MICE INDUCES
METASTATIC DISEASE.

3.1. Introduction

Given the close correlation between neu (HER/c-erbB-2) overexpression and mammary carcinogenesis, our laboratory has been interested in directly testing the tumorigenic potential of the neu oncogene in the mammary epithelium of transgenic mice. Initially, this was accomplished by generating several lines of transgenic mice carrying the activated rat neu gene under the transcriptional control of the MMTV promoter/enhancer (Muller et al., 1988; Bouchard et al., 1989). In several strains of MMTV/activated neu mice, early onset of transgene expression in the mammary epithelium was associated with the synchronous development of tumors involving the entire mammary epithelium. These results suggested that expression of activated neu requires few, if any, additional genetic events to transform the mammary epithelial cell (Muller et al., 1988).

These studies suggested that the activated neu oncogene can act as a potent oncogene in the mammary epithelium. However, overexpression of the wild-type Neu protein may be the primary mechanism contributing to breast cancer, since examination of primary breast cancer biopsies has thus far
failed to reveal comparable activating mutations (Slamon et al., 1989; Lemoine et al., 1990).

In this chapter, I directly tested the oncogenic potential of the wild-type Neu protein in the mammary epithelium of transgenic mice by establishing six lines of transgenic mice carrying a MMTV/unactivated neu fusion gene. Overexpression of the unactivated Neu product in the female mammary epithelium of five of these lines resulted in appearance of focal mammary tumors that metastasized with high frequency. These observations support the hypothesis that elevated expression of the unactivated neu associated kinase activity in the mammary epithelium induces metastatic disease.

3.2. Results

3.2.1. Expression of the Transgene Correlates with Tumor Development.

To test the oncogenic potential of the unactivated neu product in the mammary epithelium, a hybrid transcription unit comprising the MMTV promoter/enhancer and neu cDNA was microinjected into mouse zygotes (Fig. 3.1). The MMTV/unactivated neu transgene is isogenic to the MMTV/activated neu described previously (Muller et al., 1988), except for the absence of the activating mutation in the transmembrane domain and the presence of a Sal I restriction endonuclease site between the neu cDNA and the SV40 polyadenylation splicing signals. A total of eight transgenic founders were generated carrying the MMTV/unactivated neu transgene.
Figure 3.1. Structure of the MMTV/unactivated neu transgene.

The unshaded region represents the sequences within the pBR322 vector backbone. The stripped portion contains the MMTV LTR derived from the plasmid pA9 (Huang et al., 1981), the filled region corresponds to an inert region derived from the original pA9 vector. The stippled region contains the cDNA encoding the unactivated neu whereas the adjacent cross hatched region contains the transcriptional processing sequences derived from the SV40 early transcription unit. Relevant restriction sites and transcription start site (indicated by the arrow) are also shown.
Of the eight strains, six passed the transgene to their progeny in a Mendelian fashion.

To assess the tissue specificity of transgene expression in these MMTV/unactivated neu transgenic mice, 10 μg of total RNA derived from 10 different tissues from both male and female carriers of the various founder strains was subjected to RNase protection with a transgene-specific probe comprising the SV40 polyadenylation/splicing signals (pASV) (Muller et al., 1988) (Fig. 3.2). Representative results from these RNase protection experiments for the MMTV/unactivated neu strains are summarized in Table 3.1. Consistent with the observations made with other transgenic strains bearing MMTV/oncogenes, high levels of transgene expression were noted in the mammary glands of female transgenic mice in five of six lines. Lower amounts of transgene transcript were also detected in other tissues such as the salivary gland, lung and male organs (semenal vesicles, testes, epididymis) after longer exposure of the autoradiograms (see Table 3.1).

Although low-level of expression of neu transcript in the mammary epithelium did not initially affect mammary gland function or development, focal mammary tumors began to appear in these strains at about 6 months of age. Histological examination of the tumors revealed focal mammary adenocarcinomas surrounded by hyperplastic mammary epithelium (Fig. 3.3.A). These tumors were composed of solid nests of pale intermediate cells that were morphologically identical to tumors associated with activated neu (Cardiff et al., 1991). In addition, transplantation of the tumor cells into the mammary fat pads of syngeneic recipients resulted in the appearance of tumors, confirming their neoplastic potential.
Figure 3.2. Tissue specificity of transgene expression.

RNA transcripts corresponding to the MMTV/unactivated neu transgene in various organs of a female of the N#202 transgenic strain. Tissue were derived from a multiparous tumor bearing animal at 240 days of age. The antisense probe used for the RNAase protection assay is directed against the SV40 component of the transgene and yields a 784 nucleotide protected fragment. Upon longer exposure of the autoradiograms, lower amounts of transgene were also detected in the salivary gland, spleen, thymus and lung.
Table 3.1. Transgene expression and onset of tumors formation in the MMTV/unactivated neu mice.

RNAse protection analysis was performed on 10 ug of total RNA isolated from a variety of organs from both male and female carriers derived from the MMTV/activated neu transgenic lines. The probe used in this analysis is directed against the SV40 component of the transgene and yields a 784 nucleotide protected fragment. Relative levels of transgene expression are indicated by + (low), ++ (intermediate) or +++ (high). M.gl.T. refers to mammary gland tumor whereas M.gl.N. represents adjacent mammary epithelium. Other tissues examined for expression of the transgene include salivary glands (Sal), lung (L), seminal vesicles (SV) testes (T) and epididymis (Ep). Although not shown in Table 3.1, additional tissues that were examined but proved negative for expression of the transgene include brain, liver, thymus, spleen, heart, and small intestine and did not express the transgene.

NA: Not Applicable; n: number of animal analysed; M.gl.: Mammary gland.
<table>
<thead>
<tr>
<th>Line</th>
<th>Sex*</th>
<th>M.gl.T</th>
<th>M.gl.N</th>
<th>Sal</th>
<th>L</th>
<th>SV</th>
<th>T</th>
<th>Epi</th>
<th>Onset of Tumor Formation (days)</th>
<th>% metastatic tumors</th>
<th>Tumor type</th>
</tr>
</thead>
<tbody>
<tr>
<td>N#169</td>
<td>F</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>337, (n=4)</td>
<td>50, (n=2)</td>
<td>M.gl. Adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>No Tumor</td>
</tr>
<tr>
<td>N#202</td>
<td>F</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>205, (n=57)</td>
<td>72, (n=41)</td>
<td>M.gl. Adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>No Tumor</td>
</tr>
<tr>
<td>N#204</td>
<td>F</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
<td>No Tumor</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
<td>No Tumor</td>
</tr>
<tr>
<td>N#510</td>
<td>F</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>367, (n=2)</td>
<td>50, (n=1)</td>
<td>M.gl. Adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
<td>No Tumor</td>
</tr>
<tr>
<td>N#721</td>
<td>F</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>261, (n=21)</td>
<td>11, (n=2)</td>
<td>M.gl. Adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
<td>No Tumor</td>
</tr>
<tr>
<td>N#732</td>
<td>F</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>268, (n=7)</td>
<td>43, (n=3)</td>
<td>M.gl. Adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>No Tumor</td>
</tr>
</tbody>
</table>

*F, female; M, male

**Percentage of tumor bearing mice over 8 months of age possessing lung metastasis
Figure 3.3. Histopathology of the MMTV/unactivated neu transgenic mice.

A. Photomicrograph of a hematoxylin/eosin-stained slide illustrating the expansible focus of solid tumor arising in the midst of a hyperplastic, dysplastic mammary gland. Note that the dysplastic mammary cells lining dilated luminal spaces do not show lipid production. This sample was obtained from a multiparous female derived from the MMTV/unactivated neu N#202 line (N#128, 210 days of age). Magnification: x48.

B. Photomicrograph of a hematoxylin/eosin-stained slide demonstrating a typical nest of metastatic mammary adenocarcinoma cells in the lung. Note that the metastasis is in solid nests which resemble those found in the primary tumors such as those illustrated in A. This sample was obtained from the lung of multiparous female derived from the MMTV/unactivated neu N#202 line (N#3202, 221 days of age). Magnification: x48
In our best characterized strain, N\#202, 50\% of female carriers developed mammary tumors by 205 days (Fig. 3.4). The appearance of tumors in this particular line was not strictly dependent on pregnancy, because virgin female transgenic mice also developed mammary tumors. Furthermore, female transgenic mice derived from the other transgenic lines (N\#169, N\#510, N\#721, and N\#732) also developed tumors, albeit with later onset (see Table 3.1). Paradoxically, male MMTV/unactivated neu carriers did not exhibit any phenotypic abnormalities.

Because overexpression of neu in human breast cancer has been implicated as an important step in tumor progression, we compared the level of transgene expression in the tumors and in the adjacent mammary epithelium. To this end, 10 ug of total RNA derived from mammary tumors and adjacent mammary epithelium isolated from female N\#202 transgenic mice was hybridized with a radiolabelled transgene-specific probe (pASV) and subjected to RNase digestion (Fig 3.5). To ensure that equal amounts of RNA were examined, an rpl32 antisense probe was also included in the hybridization reactions. Representative results for several sets of matched tumor and adjacent mammary epithelium for the N\#202 line are shown in Fig. 3.5. Densitometric measurement of these autoradiograms revealed that many of the tumors (n=16) expressed higher levels (10 to 50 fold) of transgene RNA than the adjacent mammary epithelium. However, several other matched sets of normal and tumor tissues (n=7) expressed equivalent amounts of neu RNA (e.g., N\#5741; Fig. 3.5). While there was some variation in transgene expression in the normal mammary epithelium, all mammary tumors examined expressed elevated levels of the neu transgene.
Figure 3.4. Kinetics of tumor occurrence of the MMTV/unactivated *neu* transgenic mice.

Comparison of the kinetics of tumor formation between female transgenic carriers bearing the MMTV/activated *neu* (NF line; Muller et al., 1988) and females carrying the MMTV/unactivated *neu* construct (N#202 line). The age at which 50% of mice were found to have tumors ($t_{50}$) and the number of mice examined ($n$) are also indicated.
Figure 3.5. RNase protection analysis of expression of the transgene RNA in tumor and adjacent mammary epithelium.

RNA from control and transgenic tissues were hybridized with probes directed to the transgene (\textit{neu}), \(\beta\)-casein, and the \textit{rpL32} ribosomal protein gene. The control tissues were isolated from lactating mammary glands (M.gl.) and lung (L) of normal female FVB mice. Transgenic tissues derived from multiparous female N\#202 carriers (N\#6261, 301 days of age; N\#5741, 342 days of age; and, N\#6144, 295 days of age) include primary mammary tumor (BT), adjacent mammary epithelium (NB) and lung metastases (LM). The 784-nucleotide protected fragment corresponds to the SV40 component of the transgene (\textit{neu}), the 205-nucleotide protected fragment for \(\beta\)-casein, and the 278-nucleotide protected fragment for the \textit{rpL32} control are indicated by arrows. All assays were conducted with 10 \(\mu\)g of total RNA.
Taken together, these observations suggest that elevated expression of *neu* may be an important step for tumorigenesis.

3.2.2. Mammary Gland-Specific Expression of *neu* Is Associated with the Induction of Metastatic Disease.

Surprisingly, a high percentage of tumor bearing MMTV/unactivated *neu* animals developed metastases to the lung (Fig. 3.3B). Histological examination of lung tissue in these affected animals revealed the presence of multiple foci of metastatic mammary adenocarcinomas lodged in pulmonary vessels (Fig. 3.3B). Like the primary mammary tumors, these metastatic lung tumors also expressed elevated levels of the *neu* transgene RNA (Fig. 3.5). The extent of metastatic involvement in these lines was particularly remarkable with respect to its penetrance. For example, in the N#202 line, 72% of the tumor-bearing mice that lived to an age of 8 months or older developed metastatic disease. Similar proportions of older tumor-bearing mice from the N#169, N#510, N#721, and N#732 lineages also developed metastatic disease (Table 3.1). Consistent with these observations, metastatic foci could also be detected in lung tissue after transplantation of the primary tumors into the fat pads of normal syngeneic recipients.

To confirm that the tumors detected in the lung were of mammary origin, RNase protection analyses with a probe specific to the mammary differentiation marker, β-casein, were performed on RNA derived from both primary and metastatic tumors (Fig. 3.5). Both the primary mammary tumors and lung metastases derived from the N#202 line expressed moderate levels of β-casein RNA. However, normal lung tissue isolated from a
nontransgenic female sibling did not express detectable amounts of β-casein RNA. Together with the histological observations, these results demonstrate that expression of unactivated neu in the mammary epithelium leads to the development of metastatic disease.

3.2.3. Induction of Mammary Tumors by neu Results in Elevated neu Tyrosine Kinase Activity.

To establish whether the elevated expression of transgene transcripts observed in the tumor tissues resulted in a corresponding increase in Neu protein, Western blot analyses with Neu specific antibodies were conducted on protein extracts of normal and neoplastic tissues. The level of Neu in the tumors was higher than in the adjacent epithelium (Fig. 3.6A). Because the transforming potential of the Neu is closely correlated with its intrinsic tyrosine kinase activity, we were interested in measuring neu kinase activity in tumor and adjacent mammary epithelium derived from female animals of the N#202 line. To accomplish this, protein extracts derived from normal and tumor tissues were subjected to in vitro kinase assays using a monoclonal antibody directed against the rat Neu protein. A prominent 185-kDa phosphorylated band was observed when extracts of mammary tumors and their derived metastases were assayed (Fig. 3.6B). By contrast, no comparable autophosphorylated species could be detected when extracts of nontransgenic control tissues or the adjacent mammary epithelium were assayed for kinase activity (Fig. 3.6B). Because Neu could readily be detected in the mammary epithelium adjacent to the tumor tissue (Fig. 3.6A), these
Figure 3.6. Expression of Neu protein and associated kinase activity in tumor and adjacent mammary epithelium.

A. Western analyses of control and transgenic tissues with a Neu specific monoclonal antibody. The control tissues were isolated from lactating mammary glands (M.g.l.) and lung (L) of normal female FVB mice. Transgenic tissues derived from multiparous female N#202 carriers (N#6261, 301 days of age; N#5741, 342 days of age; and, N#6144, 295 days of age) include primary mammary tumor (BT), adjacent mammary epithelium (NB) and lung metastases (LM). The 185-kDa Neu protein is illustrated by the arrow.

B. In vitro kinase activities of the same samples as in A. The protein extracts were incubated with a Neu specific monoclonal antibody, and the immuno-complexes were subsequently labelled with $[\gamma^{32}P]ATP$. The 185-kDa Neu phosphorylated species is indicated.
observations indicate that neu-induced tumors possess higher neu-associated tyrosine kinase activity than the adjacent mammary epithelium.

3.2.4. Induction of Mammary Tumors by neu Results in the Appearance of Several Phosphotyrosine Containing Proteins.

Inspection of the phosphotyrosine containing protein profile with antiphosphotyrosine antibodies revealed that the mammary tumors derived from the MMTV/unactivated neu N#202 line expressed two prominent phosphotyrosine containing proteins of 185 kDa and 56 kDa which were not detected in the adjacent mammary tissue (Fig. 3.7B). These proteins comigrated with similar set of proteins derived from an activated neu transformed cell line (B104) (Figure 3.7A). Because the phosphorylated 185 kDa species comigrates with the predicted molecular weight of neu this protein likely represents the autophosphorylated form of the neu. When larger amounts of protein extract are examined by this approach, other, less prominent bands are also observed. These proteins migrate around 120 kDa, 62 kDa and 40 kDa. To determine whether the appearance of these tyrosine phosphorylated proteins was specific to Neu expressing tumors, we also examined the phosphotyrosine protein content of mammary tumors expressing the polyomavirus (PyV) middle T associated tyrosine kinase (MT#5524). The middle T tumors showed no evidence of the 185 kDa protein but rather possessed major tyrosine phosphorylated species migrating at 81 kDa and 56 kDa respectively (Figure 3.7B). Because PyV middle T antigen is autophosphorylated on tyrosine residues and has a predicted molecular weight of 56 kDa, this protein could be identical to that observed in the neu
Figure 3.7. Induction of mammary tumors correlates with elevated Neu expression and the induction of several tyrosine containing proteins.

A. Immunoprecipitation/western analyses of tumor and adjacent tumor tissues derived from multiparous female N#202 transgenic mice (N#2745, 147 days of age and N#445, 183 days of age) were performed with Neu specific monoclonal antibody. Also included are a positive control line B104 (Stern et al., 1986) which expresses the activated Neu protein and a negative control NIH 3T3 cell line. The 185 kDa Neu protein is illustrated by the arrow.

B. Western analyses of an identical set of samples with a rabbit antiphosphotyrosine antibody. The prominent 185 kDa and 56 kDa phosphotyrosine containing proteins are indicated.
tumors or alternatively PyV middle T product itself. Other less abundant species, including the 120 kDa, 62 kDa and 40 kDa phosphotyrosine proteins appear to be present in both middle T antigen and Neu expressing tumors upon longer exposure of the autoradiogram. However, the identity of these phosphotyrosine containing proteins remains to be established. Together, these observations suggest that deregulation of the neu tyrosine kinase activity is a pivotal step in tumor progression.

3.2.5. Detection of Mutations within the MMTV/unactivated neu transgene during mammary tumorigenesis.

Because activation of neu can occur through mutation of a single amino acid in the transmembrane domain of the Neu protein (Bargmann et al., 1986b), we investigated whether induction of mammary tumors in the MMTV/unactivated neu could occur by this mechanism. By using reverse transcriptase/polymerase chain reaction (RT/PCR) with RNA derived from neu induced tumor or adjacent mammary epithelium, we amplified cDNA encoding the transmembrane region and hybridized these DNA products to radiolabelled oligonucleotides bearing either wild type or mutant transmembrane sequences. Preliminary results from these analyses revealed that 3 of 20 tumors samples hybridized to both mutant and wild type oligonucleotides (see Fig. 3.8 for representative results). Significantly, the PCR product derived from matched adjacent mammary epithelium or the 17 other tumors failed to hybridize to the mutant oligonucleotide. This suggest that this mutation can occur in a small percentage of neu induced tumors, and that these tumors are heterogeneous, containing cells
Figure 3.8. Detection of somatic mutations within the MMTV/unactivated neu mammary tumors.

Representative results from reverse transcriptase/polymerase chain reaction (RT/PCR) analyses of RNAs derived from adjacent mammary epithelium (NB), mammary tumors (BT) from the MMTV/unactivated neu N#202 transgenic strain. Duplicate samples of the 474 base pair PCR products spanning the transmembrane domain of neu were either probed with radiolabelled oligonucleotides specific to the wild type neu transmembrane domain (1306) or mutant neu transmembrane domain (1307) using the protocol and oligonucleotides described by Bargmann et al., 1986b. Also included as controls are the RT/PCR products derived from RNA from mammary tumors from the MMTV/activated neu transgenic mice (CR#438 BT) (Muller et al., 1988) and normal mammary gland (Normal FVB Mgl). A single tumor PCR sample (#2385 BT) hybridizes to both the mutant and wild type oligonucleotides.

Oligonucleotide 1306: wildtype transmembrane domain of neu corresponding to nucleotides 5'-ACG CCC ACT ACA GTT GCA AT-3'. Oligonucleotide 1307: mutant transmembrane domain of activated neu corresponding to nucleotides 5'-ACG CCC TCT ACA GTT GCA AT-3'.
harbouring both the wild type and mutant neu alleles. Sequence analysis of one of these PCR products revealed that it possesses the same point mutation in the transmembrane domain as the one observed in the activated version of neu (Bargmann et al., 1986a). These observations raise the intriguing possibility that point mutations may play a significant role in activating neu tyrosine kinase.

3.3. Discussion

Our observations provide the first direct evidence that overexpression of the proto-oncogenic form of neu results in a heritable development of metastatic mammary tumours. In five independent strains of MMTV/unactivated neu mice (N#202, N#169, N#510, N#721 and N#732), expression of the transgene resulted in the appearance of focal mammary adenocarcinomas after long latency. In the majority of the mammary tumors analysed, the transformed phenotype was correlated with overexpression of the neu transgene, an increase in neu associated tyrosine kinase activity and the appearance of several tyrosine phosphorylated proteins. These data support the notion that the overexpression of neu and/or activation of neu results in the production of metastatic mammary adenocarcinomas.

Overexpression of neu has been frequently observed in human primary breast cancers (Slamon et al., 1989) as well as derived cell lines (King et al., 1985; Kraus et al., 1987; van de Vijver et al. 1987). In a large percentage of these human samples, overexpression of neu was associated with gene
amplification. Consistent with these clinical observations RNase protection
and Western blot analyses of normal mammary tissue and tumor tissue (Fig.
3.5, 3.6A and 3.7A) also revealed evidence of overexpression of neu in
mammary tumors. Densitometric quantitation of the levels of neu RNA in
tumor tissues revealed that the extent of overexpression varied from 10 to 50
fold. However, unlike human breast cancer samples, these tumors exhibited
no evidence of amplification of the transgene or endogeneous neu gene (data
not shown). Conceivably, an increased transcription rate or an increase in
mRNA stability of the transgene product could account for neu
overexpression. Indeed, several human mammary tumor cells overexpress
neu in the absence of detectable gene amplification (Kraus et al., 1987).

Consistent with these observations, previous studies have
demonstrated that transgenic mice expressing the activated neu oncogene in
the mammary epithelium also develop mammary tumors (Muller et al.,
1988; Bouchard et al., 1989). However, malignant progression in several of
the strains carrying the MMTV/activated neu differ from the process
observed in the MMTV/unactivated neu transgenic mice. Mammary gland-
specific expression of activated neu in several of these strains resulted in the
synchronous appearance of multifocal mammary tumors in both sexes
(Muller et al., 1988). These studies suggested that expression of activated neu
was sufficient for mammary tumorigenesis. By contrast, expression of
unactivated neu is not sufficient since mammary epithelium adjacent to
tumors expresses appreciable levels of neu protein (Fig. 3.6A). These
observations argue that additional events are involved in neu mediated
tumorigenesis. It is conceivable that differences in the activity of the neu
tyrosine kinase in the mammary epithelium of transgenic mice carrying
either the activated or unactivated neu transgene accounts for this phenotypic
variation. Consistent with this hypothesis is the observation that the neu
induced tumors possess elevated tyrosine kinase activity by comparison to
adjacent mammary epithelium (Fig. 3.6B). Unlike the activated neu
transgene which is constitutively active, activation of the wild type neu
kinase may require additional events. For example, overexpression of neu
receptor or its ligand could render neu kinase activity constitutive.
Alternatively, neu kinase activity might be influenced by mutation or by the
action of other cellular proteins. In this regard, it is interesting to note that a
small percentage of mammary tumors had acquired the activating mutation
in the transmembrane domain which renders neu kinase constitutive.

Consistent with the contention that elevated neu tyrosine kinase
activity is required for tumor formation, a number of phosphotyrosine
containing proteins including proteins of 185 kDa and 56 kDa could be
specifically detected in tumor tissues (Fig. 3.7B). The 185 kDa protein likely
represents the autophosphorylated form of the neu growth factor receptor
since in vitro kinase analyses had demonstrated elevated neu kinase activity
in tumor tissue. Interestingly, a 56 kDa phosphotyrosine protein has been
recently demonstrated to be associated with the neu receptor following
exposure to neu specific monoclonals (Scott et al., 1991). Because these
monoclonal reagents appear to mimic the expected effects of ligand
stimulation such as receptor phosphorylation and internalization, the
appearance and phosphorylation of this 56 kDa species is likely to be
important in neu signal transduction. Recent observations have shown that
this 56 kDa protein copurifies with the phosphatidylinositol 4' (PI-4) kinase which is involved in the generation of lipid secondary messengers (Scott et al., 1991; Peles et al., 1992). Alternatively, this 56 kDa protein could be Shc which is known to be tyrosine phosphorylated by Neu (Segatto et al., 1990). Together these observations suggest that mammary tumors possess elevated neu kinase activity that results in the induction of several phosphotyrosine containing cellular proteins that may be involved in neu signal transduction.

The unexpected finding that many of the older tumor-bearing neu transgenic animals developed pulmonary metastases may have important clinical implications. The observation that overexpression of neu in human breast cancer is associated with poor clinical outcome in node-negative women (Gullick et al., 1991; Paterson et al., 1991) and the results of these transgenic experiments suggest that overexpression of neu can confer an enhanced metastatic potential upon the mammary tumor cell. The metastatic foci observed in the MMTV/unactivated neu transgenic mice appear to be restricted to the lung. Because most of these metastatic foci still retain the capacity to express mammary markers such as β-casein (Fig. 3.5), it is likely that these tumors originate from the primary mammary tumor. While pulmonary metastases were observed in transgenic strains carrying the MMTV/activated neu transgene, metastasis was a relatively infrequent event (Muller et al., 1988). However, because these activated neu tumors involve the entire mammary epithelium and thus considerably shorten the animals survival, it is conceivable that the further steps necessary for metastatic progression do not have sufficient time to occur. Indeed, metastases in the
MMTV/unactivated \textit{neu} lines is observed only in older tumor bearing animals (Table 3.1).

The high penetrance of metastatic disease observed in these lines contrasts with other transgenic tumor models where metastasis is relatively rare (Pattengale et al., 1989). The high incidence of metastatic disease in these transgenic strains may reflect the ability of these associated tyrosine kinases to modulate the activity of genes involved in metastatic progression.
CHAPTER 4
INDUCTION OF MAMMARY TUMORS BY EXPRESSION OF POLYOMAVIRUS MIDDLE T ONCOGENE: A TRANSGENIC MOUSE MODEL FOR METASTATIC DISEASE.

4.1. Introduction

Transgenic mouse strains that express activated oncogenes in a variety of tissue types have been generated by a number of laboratories (reviewed in Cory and Adams, 1988; Hanahan, 1988). Although many of these strains develop heritable malignancies, both the kinetics and apparent clonal nature of these tumors argue that additional genetic events are required for the cell to acquire the full malignant phenotype (Hunter, 1991). By contrast to these observations, several transgenic strains uniformly expressing the activated neu tyrosine kinase under the transcriptional control of the MMTV LTR develop adenocarcinomas involving the entire mammary epithelium (Muller et al., 1988). Because these tumors arise synchronously and are polyclonal in origin, it was concluded that the expression of the activated neu protein was sufficient for transformation of the primary epithelial cell. These observations suggest that expression of activated neu tyrosine kinase at sufficient levels in the mammary epithelium can obviate the requirement for additional genetic alterations.

Another potent tyrosine kinase activity that has been implicated in the genesis of murine mammary tumors is that associated with the PyV middle T

83
antigen. Infection of newborn or nu/nu adult mice with PyV results in the formation of a number of epithelial and mesenchymal tumor types of which mammary adenocarcinomas represent a significant proportion (Dawe et al., 1987; Berebhi et al., 1988; 1990; Rassoulzadegan et al., 1990). Genetic analyses of polyomavirus mediated tumorigenesis has shown that a functional middle T antigen is required for tumor induction (Israel et al., 1979).

The potent transforming activity of middle T antigen is dependent on its association with a number of cellular proteins. For example, there is compelling evidence that middle T specifically associates with and activates the tyrosine kinase activity of a number of c-src family members (c-src, c-yes, and fyn) (Bolen et al., 1984; Kornbluth et al., 1986; Courtneidge and Hebner, 1987; Cheng et al., 1988; Kypta et al., 1988). Furthermore, formation of this complex appears to be critical for middle T antigen to transform cells (Cook and Hassell, 1990). In addition to association with the src family of tyrosine kinases, middle T antigen is also known to interact with the 85 kDa subunit of the phosphatidylinositol 3'-kinase (Whitman et al., 1985; Talmage et al., 1989) and this association is also required for its transforming activity (Talmage et al., 1989). More recently, stable complexes between protein phosphatase subunits A (regulatory) and C (catalytic) and middle T antigen have also been detected (Pallas et al., 1990; Walter et al., 1990) but the role of this complex in tumorigenesis is still unknown.

Because of the ability of PyV middle T antigen to potentially signal cell proliferation through a number of signal transduction pathways and its likely capacity to induce mammary adenocarcinomas, I was particularly interested in studying its oncogenic potential in the mammary gland. To accomplish
this, I directed the expression of the middle T antigen to the mammary epithelium by isolating transgenic mice carrying a MMTV/PyV middle T antigen fusion gene. Expression of middle T antigen in several independent transgenic strains resulted in synchronous appearance of multifocal tumors involving all mammary glands. Thus, expression of the middle T oncogene appears to result in rapid tumor progression of the mammary epithelium. Interestingly, many of the middle T transgenic mice developed multiple metastases in the lung. The multifocal nature of these tumors and the high incidence of metastatic disease observed in these strains have important implications for understanding the molecular basis of tumor progression.

4.2. Results

4.2.1. Generation of MMTV/PyV Middle T Antigen Mice and Tissue Specificity of Transgene Expression.

To derive transgenic mice expressing PyV middle T antigen in the mammary gland, a cDNA encoding PyV middle T antigen (Treisman et al., 1981) was inserted into an MMTV LTR expression vector (Fig. 4.1). The MMTV component was derived from plasmid PA9 (Huang et al., 1981), whereas the SV40 transcriptional processing signals at the 3' end of the cDNA were obtained from plasmid CDM8 (Seed et al., 1987). To ensure that the MMTV/middle T antigen recombinant was biologically active, the transforming potential of the fusion gene was first assessed by transfection
Figure 4.1. Structure of the MMTV/PyV middle T antigen transgene.

The unshaded region represents the sequences within the Bluescript vector backbone, the stripped portion contains the MMTV LTR derived from the plasmid pA9 (Huang et al., 1981), the filled region corresponds to an inert region derived from the original pA9 vector, the stippled region contains the cDNA encoding the polyomavirus middle T antigen whereas the adjacent crosshatched region contains the transcriptional processing sequences derived from the SV40 early transcription unit. Relevant restriction sites and transcription start site (indicated by the arrow) are also shown.
into Rat-1 cells. As expected, this construct was capable of transforming Rat-1 cells in the presence of supplemented glucocorticoids. Before this plasmid was microinjected into one-cell mouse embryos, plasmid sequences were released by digestion with SalI and SpeI (Fig. 4.1). After injection of this construct into mouse zygotes, seven transgenic founder animals (MT#121, MT#196, MT#235, MT#634, MT#654, MT#668, and MT#670) were generated. With the exception of the two founder animals MT#235 and MT#196, both of which failed to transmit the transgene, the founders passed the transgene to their progeny in a mendelian fashion.

To assess the tissue specificity of transgene expression, 10 µg of total RNA isolated from a variety of tissues was subjected to RNase protection using a transgene-specific probe corresponding to the 5' portion of the PyV middle T cDNA, yielding a 203-nucleotide protected fragment. To ensure that equal amounts of RNA of each samples were analysed, an antisense probe derived from the mouse ribosomal protein rpl32 (Dudov and Perry, 1984) was included in each hybridization reaction. Representative results of these RNase protection analyses are shown for the MMTV/PyV middle T antigen MT#634 line in fig. 4.2. Both male and female carriers derived from this line developed extensive mammary tumors with early onset (Table 4.1). Female transgenic mice expressed high levels of the transgene product in the mammary tumors, with lower levels detected in the ovaries and salivary glands. Interestingly, in older (2 to 3 months) female transgenic animals, middle T transcripts were also detected in the lungs (Fig. 4.2). This lung-specific expression was not observed in younger animals and is correlated
Figure 4.2. Tissue specificity of transgene expression in the MMTV/PyV middle T antigen transgenic mice.

RNA transcripts corresponding to the MMTV/middle T transgene in various tissues of the MT#634 transgenic strain. Tissue were derived from a multiparous MT#634 female at 119 days of age (MT#5258) and an MT#634 male at 119 days of age (MT#2833). The antisense probe used in this RNAase protection analysis (shown at the bottom) protects a 203-nucleotide fragment marked by MT and an arrow. Nucleotide numbers refer to polyomavirus early region nucleotide sequence (Soeda et al., 1980). Also shown is the RNAase protection analysis with an antisense probe directed against the mouse rPL32 ribosomal gene. The rPL32 probe protects a 278-nucleotide fragment and is marked by L32 and an arrow. A lower band is also consistently observed in these RNAase protections with the L32 probe.
RNAase protection analysis was performed on 10 µg of total RNA isolated from a variety of organs from both male and female carriers derived from the MMTV/PyV middle T transgenic lines. The probe used in the analysis is directed against the 5'end of PyV middle T cDNA and yields a 203-nucleotide protected fragment. Relative levels of transgene expression are indicated by + (low), ++ (intermediate), or high (+++). M.gl., mammary gland; Sal, salivary gland; L, lung; O, ovary; SV, seminal vesicle; T, testes; and Epi, epididymis. Also, NA refers to not applicable; ND to not determined; and n to number of animals analyzed. Unless indicated, all transgenic mice analyzed were tumor bearing.
<table>
<thead>
<tr>
<th>Lines</th>
<th>Sex</th>
<th>M.gl.</th>
<th>Sal</th>
<th>L</th>
<th>O</th>
<th>SV</th>
<th>T</th>
<th>Epi</th>
<th>Onset of tumor formation (days)</th>
<th>Tumor types</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT#121</td>
<td>F</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>94 ± 18, (n=20) NA</td>
<td>M.gl. Adenocarcinoma no tumor</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>MT#196</td>
<td>M</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++ ND ND</td>
<td></td>
<td></td>
<td></td>
<td>25, (n=1)</td>
<td>Adenocarcinoma of M.gl. and sem. vesicles, Hemangiomas</td>
</tr>
<tr>
<td>(Founder)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT#235</td>
<td>F</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>70, (n=1)</td>
<td>M.gl. Adenocarcinoma</td>
</tr>
<tr>
<td>(Founder)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT#634</td>
<td>F</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>34 ± 6, (n=35) NA</td>
<td>M.gl. Adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td>+</td>
<td></td>
<td>83 ± 20, (n=20)</td>
<td>M.gl. Adenocarcinoma</td>
</tr>
<tr>
<td>MT#654</td>
<td>F</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>175 ± 68, (n=4) NA</td>
<td>M.gl. Adenocarcinoma no tumor</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>MT#668</td>
<td>F</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>36 ± 2, (n=30)</td>
<td>M.gl. Adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td></td>
<td>+</td>
<td></td>
<td>70, (n=20)</td>
<td>M.gl. Adenocarcinoma</td>
</tr>
<tr>
<td>MT#670</td>
<td>F</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>155, (n=3)</td>
<td>M.gl. Adenocarcinoma no tumor</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>
with the appearance of multifocal lung metastases. Male transgene carriers expressed high levels of the fusion gene in mammary tumors and lung metastases, whereas lower levels were detected in the salivary glands and epididymis.

The tissue specificity of transgene expression was also assessed for the remaining six transgenic lines of MMTV/middle T antigen mice by using the same RNase protection probe. As shown in Table 4.1, variable levels of transgene expression were noted in mammary glands of female transgenic mice derived from the MT#121, MT#654, MT#668, and MT#670 lines. Among the different female transgenic animals, considerable variation in both the amount and the temporal pattern of transgene expression was observed. For example, transgene transcripts were readily detected in the mammary glands derived from virgin female carriers of the MT#634 and MT#668 lines. By contrast, at least two pregnancies were required in order to detect similar levels of transgene expression in the MT#121, MT#654, and MT#670 strains (Table 4.1). As observed with the other transgenic strains, the appearance of these tumors was strictly correlated with the expression of the transgene. With the possible exception of the MT#196 transgenic founder, which developed mammary tumors, a seminal vesicle neoplasm, and hemangiomas, the lower amounts of middle T antigen RNA observed in the various tissues were not associated with any other apparent growth disturbance.
4.2.2. The MMV/PyV Middle T Antigen-Induced Mammary Tumors Possess Associated Tyrosine Kinase Activity.

To establish whether the elevated expression of transgene transcript observed in the tumor tissues resulted in a corresponding increase in middle T protein level, Western blot analysis, using MT-specific polyclonal antisera, was conducted on tumor extracts obtained from the MT#634 transgenic line. As observed in figure 4.3, both species, the 56 and 58 kDa of middle T antigen could be detected in the tumors of these transgenic animals as well as in middle T transformed cell line (Fig. 4.3). To confirm that the detected transcripts encoded a functional middle T antigen, tissue extracts derived from the mammary glands of several of these transgenic lines were subjected to an in vitro kinase assay using a polyclonal antisera directed against middle T antigen. As a consequence of PyV middle T antigen's ability to associate with and activate a number of c-Src tyrosine kinases, the middle T protein becomes autophosphorylated on tyrosine residues in vitro (Courtneidge and Smith, 1983; Bolen et al., 1984; Kornbluth et al., 1986; Kypta et al., 1988; Cheng et al., 1988). As illustrated in Fig. 4.4, a prominent 56-kDa phosphorylated band was observed in lanes incubated with the middle T-specific antisera. Because the band observed in the tumor extracts comigrated with middle T antigen derived from a Rat-1 cell line expressing middle T antigen, these observations suggest that the tumor extract possesses middle T-associated kinase activity. Incubation of the extracts with a nonspecific control antibody (mouse immunoglobulin G) resulted in the appearance of a background phosphorylated band that is present in all lanes. Together, these results
Figure 4.3. Western immunoblot analyses of mammary tumor derived from the MMTV/PyV middle T antigen mice.

Western analyses of control and transgenic tissues with a middle T-specific polyclonal antibody. Mammary tumor extract derived from multiparous female carriers MT#634 (founder animal, 90 days of age) and F1 progeny (MT#1961, 95 days of age) were analysed for the presence of middle T protein. As controls, an adenovirus middle T transformed cell line (MT-293) (Davidson and Hassell) was examined along with mammary gland tissue (Normal Breast) isolated from normal female FVB mice. The 56-58 kDa middle T protein is indicated by MT and arrow.
Figure 4.4. Mammary tumors derived from the MMTV/PyV middle T antigen strains possess middle T associated tyrosine kinase activity.

*In vitro* kinase activities of mammary tumor extracts derived from multiparous female MT#634 (MT#5524, 76 days old), MT#121 (MT#765, 154 days old), and MT#668 (MT#5532, 85 days old) carriers incubated with polyclonal rat antiserum directed against middle T antigen (+) or nonspecific antibody (-). Also included are a negative control with Rat-1 (RAT) fibroblasts and a positive control with middle T-transformed Rat-1 (MT-RAT) fibroblasts. The 56-kDa phosphorylated middle T antigen is indicated at the left (MT).
indicate that the MMTV/middle T transgene in these strains associates with an active tyrosine kinase in the mammary epithelium.

4.2.3. Expression of the PyV Middle T Antigen in the Mammary Epithelium Results in the Generation of Multifocal Mammary Tumors.

Elevated expression of middle T antigen in the mammary glands of transgenic mice had dramatic consequences. In three of the five characterized transgenic lines, high levels of transgene expression were initially associated with the inability of female carriers to nurse their young. In addition, the MT#235 founder animal displayed an inability to lactate. In two of these transgenic lines (MT#634 and MT#668), this phenotype was apparent during the initial pregnancy, but the MT#121 strain demonstrated the nursing defect only after multiple pregnancies. Although there was some variation between these strains with respect to appearance of this phenotype, the inability to nurse was closely correlated with the onset of transgene expression. By comparison with virgin female normal mammary tissue (Fig. 4.5A), whole-mount examination of virgin female mammary tissue from the MT#634 strain (3 weeks of age) revealed the presence of multiple mammary adenocarcinomas (Fig. 4.5B). These tumors were generally highly fibrotic, with dense connective tissue separating individual nests of tumor cells (Fig. 4.5C). By 5 weeks of age, all female carriers from the MT#634 (n=35) and MT#668 (n=4) lines had developed palpable mammary tumors (Table 4.1) that involved the entire mammary fat pad. The multifocal appearance of mammary tumors in these strains was not dependent on pregnancy, because
Figure 4.5. Histopathology of MMTV/FyV middle T transgenic mice.

A. Photomicrograph of a hematoxylin/eosin-stained whole mount of the mammary fat pad of a wild type virgin mouse at 3 weeks of age showing normal growth and development. Magnification, x16.

B. Photomicrograph of a hematoxylin/eosin-stained whole mount of the mammary fat pad of an MT#634 virgin transgenic female (MT#907) at 21 days of age. Compare with panel A. Note the irregular formation of side branches, enlarged terminal buds and two large multilobular tumor masses (arrows). Magnification, x16.

C. Photomicrograph of a sclerosing mammary adenocarcinoma from a middle T transgenic multiparous female mouse (MT#634 at 110 days of age). Note the dense connective tissue separating the attenuated cords of poorly differentiated mammary tumor cells. This pattern is typical of these transgenic mice. Magnification, x87.

D. Photomicrograph of the lungs of the same mouse showing multiple metastases. Note that the tumor cells form well-defined acinar structures with very little stroma separating the epithelium. Also note that the tumor cells are intra-aveolar rather than intravascular, indicating growth outside of the vessels. Magnification, x87.
virgin female carriers displayed an identical tumor phenotype. The appearance of mammary tumors in the MT#121 line was closely correlated with the delayed onset of transgene expression, where 50% of female carriers at risk developed tumors by 94 days (Table 4.1). Despite the delayed kinetics of tumor formation, all multiparous MT#121 female carriers developed mammary tumors that eventually involved the entire mammary fat pad.

Male transgenic mice (n=17) derived from the MT#634 strain also developed mammary adenocarcinomas with 100% penetrance, albeit with delayed onset (Table 4.1). The appearance of mammary tumors in male transgenic mice is consistent with results obtained with both male MMTV/v-Ha-ras and MMTV/activated neu transgenic mice (Sinn et al., 1987; Muller et al., 1988) and may result from expression of the oncogene in the male mammary epithelium prior to its normal regression. By contrast, male transgenic mice derived from the MT#121, MT#654 and MT#670 strains did not develop mammary tumors, perhaps because of delayed onset of transgene expression. Both the rapid kinetics and the global nature of the tumor phenotype exhibited by these MMTV/middle T antigen transgenic mouse strains suggest that expression of middle T antigen at appropriate levels can lead to transformation of the mammary epithelium.

4.2.4. The Middle T Oncogene Induces Metastatic Disease.

As shown in Table 4.1, transgene expression was noted in the lung tissue of older individuals derived from the MT#121, MT#634, MT#654, MT#668, and MT#670 lines. Histological examination of lung tissue derived from MT#121, MT#634, MT#654, and MT#668 transgenic mice revealed the
presence of multiple foci of metastatic mammary adenocarcinomas lodged in the lung parenchyma (Fig. 4.5D and 4.6A). By contrast to the primary mammary tumors, the pulmonary metastases contained little or no connective tissue separating nests of tumor cells (compare Fig. 4.5C and 4.5D). Because lung tissue was not obtained from MT#196 and MT#235 founder animals, it was not possible to assess whether middle T antigen expression observed in the lung was the result of metastatic disease. The extent of metastatic involvement in these lines was particularly remarkable with respect to both its degree and penetrance (Fig. 4.5D and 4.6A). For example, in the MT#634 strain, 94% of tumor-bearing females developed metastatic disease by 3 months of age (Table 4.1). Male MT#634 tumor-bearing animals also developed metastatic disease, albeit with lower penetrance (80%). Similar proportions of the MT#121 (90%) and MT#668 (100%) tumor-bearing animals also developed metastatic disease during a 3-month observation period (Table 4.1). Consistent with these observations, metastatic foci could be detected in either the lymphatic or the lung tissue after transplantation of the primary tumors from the tumor-bearing MMTV/middle T antigen transgenic animals into the fat pads of normal syngeneic recipients.

While these histological observations strongly suggest that the tumors in the lung were of mammary origin, further molecular analyses with mammary gland-specific probes were performed to establish this point. The metastatic nature of these lung tumors was confirmed by assessing whether these tumors were capable of expressing mammary differentiation markers such as β-casein. Using a probe directed to the 5'end of the milk gene β-casein, RNase protection experiments were conducted on total RNA derived
Figure 4.6. The expression of the middle T oncogene results in metastatic mammary adenocarcinomas.

A. Lung tissue isolated from both MT#634 and FVB control animals. Note the extensive metastatic mammary tumors located throughout the lung tissue of the multiparous MT#634 female carrier (MT#5579) at 122 days of age.

B. RNAase protection with control and transgenic tissues with probes directed to β-casein, middle T and the rPL32 ribosomal internal control. The control tissues were isolated from the mammary glands of virgin FVB, 4-day and 12-day pregnant mice, as well as from normal lung tissue (lane L). Transgenic tissues derived from multiparous female MT#634 (MT#5579, 122 days of age), MT#121 (MT#5183, 130 days of age), and MT#668 (MT#5532, 85 days of age) carriers include primary breast tumors (lane BT) and corresponding lung metastases (lane LM). The 205-nucleotide protected fragment for β-casein, the 203-nucleotide protected fragment for middle T transcript (MT) and the 278-nucleotide protected fragment for the rPL32 ribosomal control are indicated by arrows.
from both primary and lung tumors (Fig. 4.6B). Both the primary mammary tumor and lung metastases from the MT#121, MT#634, and MT#668 lines expressed moderate levels of β-casein transcripts. By contrast, RNA derived from normal lung tissue was completely devoid of any detectable β-casein mRNA. Taken together with the histological observations, these results demonstrate that expression of middle T antigen in the mammary epithelium leads to the development of metastatic disease.

4.2.5. Elevated Proteolytic Activity in MMTV/PyV Middle T Induced Mammary Tumors is Associated with Metastatic Progression.

The development of metastatic disease is frequently associated with the ability of the tumor cells to express a variety of proteolytic enzymes including stromelysin and collagenases (Liotta et al., 1991). To determine whether this was true for the tumors derived from the MMTV/middle T transgenic mice, we subjected tumor extracts to zymographic analyses. Briefly, 100 μg of protein extract was electrophoresed through SDS-acrylamide gel embedded with gelatin and the presence of protease was detected after renaturation as clear area in the gelatin substrate following staining with coomassie blue. To ensure equal loading of protein, the gelatin-gels were then destained for comparison. By contrast to the normal mammary controls, prominent zones of proteolysis (visualize as clear band in fig. 4.7A) were observed at 50 and 90 kDa in mammary tumors derived from the MT#634, MT#668 and MT#121 transgenic line. Interestingly, these protease activities migrate with the expected size of the human 92 kDa collagenase type IV and murine 50 kDa
Figure 4.7. Evidence of elevated proteolytic activity in the MMTV/PyV middle T induced mammary tumors.

A. Zymographic analysis of the primary tumors and corresponding lung metastases of MMTV/middle T antigen transgenic mice. The control tissues were isolated from the mammary glands of virgin normal FVB mice (virgin M.g.l.) and 12-day (lactating M.g.l.)-pregnant mice, as well as from normal lung tissue (Lung). Transgenic tissues derived from multiparous female MT#634 (MT#5579, 122 days of age), MT#121 (MT#5183, 130 days of age), and MT#668 (MT#5532, 85 days of age) carriers include primary breast tumors (lane 1°tumor) and corresponding lung metastases (lane Lung Met.). The elevated proteolytic activity (92 kDa and 50 kDa species) are indicated on the right by the arrows.

B. Northern blot analysis of primary breast tumor (BT) and corresponding lung metastasis (LM) probed with the mouse stromelysin. The tissues (control and transgenic) were derived from the same source as described above. The control, β2-microglobulin, was also added to the hybridization reaction.
stromelysin or 50 kDa collagenase (Matrisian et al., 1990). In control tissues derived from either virgin and pregnant mammary epithelium, or from other mammary tumor type (activited neu), these bands of proteolysis activity were either not present or observed at reduced intensity. The observation that all protease activities were inhibited by chelators such as EDTA argues that these proteolytic species are members of the metalloproteinase family. Surprisingly, zymographic examination of extracts derived from lung metastases did not exhibit any evidence of enhanced proteolytic activity relative to the normal lung (Fig. 4.7A). In particular, the 50 kDa species was dramatically reduced in the metastatic tumors by comparison to the primary tumor cell. Because the level of middle T antigen produced between the primary and the metastatic tumors were approximately equal (Fig. 4.6B), these observations cannot be simply accounted by a variation in the amount of tissue examined. Rather these results suggest that the difference in proteolytic activity displayed by the primary and metastatic tumors is the result of the local tissue environment.

Because the 50 kDa protease species was close to the predicted molecular weight of mouse stromelysin, we decided to assess by Northern blot hybridization whether this protease was aberrantly expressed. Mouse stromelysin transcripts were readily observed in tumors originating from three independent strains of MMTV/middle T antigen mice (MT#634, MT#121, MT#668) (Fig. 4.7B). Stromelysin transcripts were not detected in either normal lung or normal mammary epithelium. However, the metastatic lungs expressed variable amount of stromelysin transcripts. For example, while the metastatic tumor derived from the MT#634 and MT#121
lines failed to express mouse stromelysin, the MT#668 pulmonary metastases expressed fairly high levels of mouse stromelysin transcript (Fig. 4.7B). Because the zymographic analysis of the metastatic tumors from all three strains exhibited reduce levels of activity of 50 kDa protease, these results imply that expression of mouse stromelysin can not solely account for this activity. Taken together, these results suggest that the enhanced proteolytic activity observed in these tumors may be due to overexpression of at least one member of the metalloproteinase family (stromelysin) and further indicate that the tissue environment has a major influence on this proteolytic activity.

4.3. Discussion

The behaviour of the PyV middle T oncogene in the mammary epithelium provides important insight into the process of malignant progression. In four independent strains of MMTV/PyV MTAg transgenic mice, expression of the transgene ultimately resulted in the uniform morphological transformation of the mammary epithelium. Virgin female transgenic mice derived from the MT#235 founder, MT#634, and MT#668 strains developed multifocal adenocarcinomas as early as three weeks of age (Figure 4.5B). Both the simultaneous occurrence and multifocal nature of the tumors in these strains suggests that middle T oncogene expression leads to rapid epithelial cell transformation.

The potent oncogenic potential of middle T antigen in the mammary gland is further supported by the results obtained with the MT#121 transgenic strain. In this particular transgenic line, mammary gland specific expression
of the middle T transgene was not detected until several pregnancies had occurred. However, once transgene expression was observed these animals developed multifocal mammary adenocarcinomas that eventually involved the entire mammary epithelium. Conceivably, the difference in the temporal kinetics of transgene expression among the various transgenic strains could be influenced by the site of integration of the transgene. For example, variation in both the spatial and temporal patterns of transgene expression were also observed in transgenic mice bearing either the MMTV/activated neu transgene (Muller et al., 1988) or an elastase promoter activated ras fusion gene (Quiafe et al., 1987). Moreover, the short latency between transgene expression and widespread morphological transformation of the mammary epithelium further argues that progression from a normal epithelial cell to a tumor cell in these mice requires few, if any, additional genetic events.

Consistent with this conclusion, previous studies of PyV middle T antigen in transgenic and chimeric mice have shown similar rapid tumor kinetics. For example, expression of the middle T oncogene under its own promoter or the MoMuLV promoter in transgenic mice results in disseminated endothelial tumors (Bautch et al., 1987; Williams et al., 1988). In the latter case, these haemaginomas resulted in embryonic lethality due to early onset of expression of middle T antigen. Because these endothelial tumors were polyclonal in nature and appeared coincident with the first appearance of yolk sac endothelial cells, it was proposed that middle T antigen acted as a single-step oncogene (Williams et al., 1988). However, because these tumors could potentially recruit normal endothelial cells to the haemaginoma, it was not clear whether all constituent cells were
morphologically transformed (Williams et al., 1989). In another set of experiments, transgenic mice expressing the middle T oncogene in neuronal and epithelial tissues resulted in the formation of multiple neuroblastomas and carcinomas (Aguzzi et al., 1990; Rassoulzadegan et al., 1990). However, because these transgenics exhibited preneoplastic lesions prior to the onset of tumor formation, additional genetic events were likely required.

The rapid tumor progression observed in the middle T oncogene transgenic mice contrasts with the observations made by a number of laboratories with transgenic mice bearing activated oncogenes. For example, multiple genetic events appear to be required for malignant progression in transgenic mice expressing oncogenes such as c-myc, v-Ha-ras or c-fos in variety of different tissue types (Brinster et al., 1984; Adams et al., 1985; Ruther et al., 1987; Sinn et al., 1987). However, it has recently been reported that one transgenic strain of mice carrying the activated neu gene under the transcriptional control of MMTV/LTR develop polyclonal mammary tumors without the need for a second event (Muller et al., 1988). It is interesting to note that both the activated neu and polyomavirus middle T oncogenes are associated with deregulated tyrosine kinase activities that are refractory to normal cellular regulation. Whether the powerful tissue specific transforming activity exhibited by these oncogenes reflects the sensitivity of the mammary epithelial cell to a common tyrosine kinase pathway awaits further analyses.

While the molecular basis for the potent transforming activity exhibited by the middle T oncogene is unclear, it is conceivable that deregulation of multiple signal transduction pathways through its association
with the src family of tyrosine kinases, the PI-3'kinase and the protein phosphatase 2A individually contribute to the overall transformed phenotype. Indeed, PyV middle T antigen molecules impaired in their ability to deregulate either of these pathways exhibit a pronounced reduction in the ability to transform cells in vitro or to induce tumors in animals (Talmage et al., 1989; Cook et al., 1990). Future experiments directed towards deregulating each of these signal transduction pathways individually in the mammary gland should allow this question to be addressed.

The unexpected finding that expression of the middle T antigen was closely associated with pulmonary metastases may provide important insight into the malignant progression. By contrast to other MMTV/oncogene bearing transgenic mice where metastasis is a relatively rare occurrence (Pattengale et al., 1989), nearly all tumor bearing MMTV/middle T transgenic carriers thus far analyzed have developed metastatic disease. It is likely that these metastatic tumors originate from the primary mammary tumors because they still retain the capacity to express mammary markers such as β-casein. Consistent with this conclusion is the observation that transplantation of these primary mammary tumors into the fat pad of syngeneic recipients frequently resulted in metastasis. The metastatic tumors were restricted to the lung and do not appear to seed in other tissue sites. These metastatic foci appear to lodge in the vessels and grow by local expansion and invasion. The apparent specificity of these metastases to the lung may simply reflect the ability of the fine capillary beds of the lung to trap tumor emboli that have entered the blood stream. Alternatively, the process of metastases, in this system, may exhibit target specificity perhaps mediated
through the expression of ligand specific cell adhesion molecules or the presence of a locally produced growth factor. Given the penetrance of metastases observed in these strains, it is conceivable that middle T is activating cellular genes that are involved in metastatic progression.

The molecular basis for middle T induced metastases appears to be correlated with an increase in the intrinsic proteolytic activity exhibited by tumor extracts. As shown by zymographic analyses, the mammary tumors express proteolytic activities which comigrate with the 92 kDa collagenase type IV and the 50 kDa mouse stromelysin or 50 kDa mouse collagenase. Indeed, Northern blot analyses showed that at least one member of the mouse stromelysin family is overexpressed in these tumors relative to normal mammary epithelia. Curiously, upon metastases to the lung, these tumors appear to lose the capacity to exhibit enhanced proteolytic activity (Fig. 17). The lower protease activities exhibited by the pulmonary metastases cannot be accounted solely by decrease levels of stromelysin expression since these transcripts can be detected in the MT#668 pulmonary tumors suggesting that other members of the mouse stromelysin and/or collagenase families are also involved in the metastatic phenotype observed in these lines. Consistent with this hypothesis, it has recently been reported that a novel stromelysin, stromelysin-3, was involved in metastasis of human breast carcinomas and is specifically expressed by adjacent stromal cells of breast carcinomas (Basset et al., 1990). In this regard, it is interesting to note that the primary mammary tumors possess an extensive stromal component relative to the secondary lung metastases. Conceivably, this additional stromal component may be responsible for the elevated proteolytic activity exhibited by the primary
tumors in comparison to their secondary metastasis. Whether expression of the middle T oncogene influences proteolytic balance directly in the tumor cells or indirectly through its action on the adjacent stroma is unclear. It is conceivable that the tumor cells stimulate the adjacent stroma perhaps through the mediation of growth factor(s) to secrete proteases. Indeed, expression of human stromelysin-3 can be induced by stimulation of human fibroblasts with a number of polypeptide growth factors (Basset et al., 1990).

Because the PyV middle T tumors exhibit elevated proteolytic activity, genes encoding the various members of the protease family and their inhibitors may be potential downstream targets of the PyV middle T associated tyrosine kinase. Indeed, the matrix metalloproteinases are frequently overexpressed in transformed cells and in tumors with metastatic potential (Matrisian et al., 1986; Matrisian and Bowden, 1990; Tandon et al., 1990; Liotta et al., 1991). In fact, endothelial cells expressing PyV middle T antigen express high levels of urokinase plasminogen activator (uPA) and low levels of its cognate inhibitor (PAI-1) (Montesano et al., 1990). Whether a similar proteolytic imbalance is responsible for the metastatic phenotype observed in the PyV middle T strains awaits further analysis.

Breast cancer is the leading cause of death among non-smoking women and most patients who die from breast cancer do so because the tumor metastasizes. A number of clinical studies have shown a close relationship between a tumor's capacity to express proteases and its metastatic potential. For example, the amplification and overexpression of proteases such as cathepsin D and stromelysin-3 are associated with decreased survival in breast cancer (Tandon et al., 1990; Basset et al., 1990). Given that the
expression of metalloproteinases can be influenced by the activation of a number of growth factor/receptor signal transduction pathways (Basset et al., 1990), it is apparent that understanding the relationship between oncogene expression and metastasis will have profound clinical implications. Indeed, the correlation seen between the overexpression of the growth factor receptors such as c-erbB-2 in human breast cancer and clinical prognosis (Slamon et al., 1987; 1989) could be the result of downstream activation of proteolytic enzymes. In this regard, the MMTV/middle T antigen transgenic mice represent an unique model system to study the molecular basis of metastasis. For example, one could use this transgenic system to elucidate the identity and mechanism of action of proteases and their regulators in mammary tumors or one could examine the extent to which they contribute to the metastatic phenotype. Moreover, the possibility exist that these transgenic mice might be employed as a useful animal model to assess the efficacy of drugs that might potentially interfere with metastatic progression. Given the clinical importance of metastasis, this transgenic model could prove useful to understand the molecular basis of metastasis and to bring about its control.
CHAPTER 5

ACTIVATION OF THE C-SRC TYROSINE KINASE IS REQUIRED FOR THE INDUCTION OF MAMMARY TUMORS IN TRANSGENIC MICE.

5.1. Introduction

The potent transforming properties of the PyV middle T antigen results from its capacity to associate with and activate a number of cellular enzymes. In addition to its ability to associate with and activate different members of the Src family (Courtneidge and Smith, 1983; Kornbluth et al., 1986, Kypta et al., 1988), PyV middle T antigen is also known to interact with the 85-kDa subunit of the phosphatidylinositol-3'-kinase (Whitman et al., 1985; Courtneidge et al., 1987), and this association is required for PyV middle T mediated tumorigenesis (Tal mage et al., 1989). Although stable complexes between protein phosphatase 2A (regulatory) and C (catalytic) and PyV middle T have also been detected (Pallas et al., 1990; Walter et al. 1990), their role in PyV middle T mediated tumorigenesis is unknown.

While it is clear that the interaction of PyV middle T antigen with these cellular proteins plays an important role in tumorigenesis, the relative contribution of each of these protein complexes to transformation remains to be defined. In this chapter, I will directly test the role of c-Src and c-Yes in PyV middle T antigen induced mammary tumorigenesis by crossing transgenic mice carrying the MMTV/PyV middle T oncogene with mice carrying either disrupted c-src or c-yes alleles (Soriano et al., 1991; Soriano, unpublished)
al., 1991; Soriano, unpublished observations). By contrast to the rapid induction of mammary tumors observed in the parental MMTV/PyV middle T transgenic strains, mammary gland-specific expression of the PyV middle T antigen in mice defective in c-Src function lead to the development of cystic hyperplasia of the mammary gland which rarely progressed to full malignancy. Significantly, transgenic mice expressing PyV middle T antigen in the mammary epithelium of the c-Yes deficient mice developed multifocal metastatic mammary tumors at rates comparable to the parental MMTV/PyV middle T strains. These observations indicate that a functional c-Src is required for PyV middle T induced mammary tumorigenesis and metastases, and that the mammary epithelium is particularly sensitive to activation of the c-Src signal transduction pathway.

5.2. Results

5.2.1. Expression of the PyV Middle T Oncogene in the Mammary Epithelium Results in Activation of c-Src and c-Yes Tyrosine Kinases.

Because PyV middle T antigen can associate with and activate a number of c-Src family members in established cell lines, I assessed which of these tyrosine kinases were activated in the PyV middle T antigen induced mammary tumors. To this end, tumor tissue extracts from several MMTV/PyV middle T antigen transgenic strains (MT#121, MT#634, and MT#668) were immunoprecipitated with either c-Src- or c-Yes-specific
monoclonal antibodies and subjected to in vitro kinase assays using acid denatured enolase as a substrate (Fig. 5.1). These experiments were conducted under conditions where incorporation of $^{32}$P isotope into enolase substrate occurs in a linear fashion (Kypta et al., 1990). A prominent phosphorylated band corresponding to enolase was observed in lanes where the protein extracts were incubated with the c-Src and c-Yes-specific antibodies (lanes marked +). On longer exposure of the autoradiograms, a band corresponding to autophosphorylated c-Src and c-Yes was also observed in tumor extracts. Incubation of these protein extracts with a nonspecific control antibody (mouse immunoglobulin G) (lanes marked -) resulted in the weakly phosphorylated enolase band. These phosphorylated bands comigrated with those observed in brain tissue which is known to express high levels of endogenous c-Src and c-Yes (Aguzzi et al., 1990; Soriano et al., 1991). Quantitative evaluation by phosphorimager analysis revealed that the tumor samples from the MMTV/PyV middle T animals had on average 5 fold greater c-Src kinase and 6 fold greater c-Yes kinase activities than the nontransgenic mammary epithelium. Although the increase in c-Src and c-Yes activities were modest, these values were consistently observed with multiple independent tumor extracts (n=9). By contrast to c-Src and c-Yes, incubation of tumor and normal mammary gland extracts with a Fyn specific antibody failed to show evidence of enhanced Fyn kinase activity in the mammary tumors (Data not shown).
Figure 5.1. Activation of the c-Src family tyrosine kinases in the PyV middle T induced mammary tumors.

(A) *In vitro* kinase activities of mammary tumor extracts derived from the different MMTV/PyV middle T transgenic strains including MT#121 (MT#742, 83 days of age), MT#634 (MT#616, 112 days of age), and MT#668 (MT#9313, 110 days of age). All tumors were isolated from multiparous female carriers. Each protein extract was immunoprecipitated with antibodies specific to c-Src, and c-Yes (lanes marked +). Normal mouse sera was used as a nonspecific control antibody (lanes marked -). In addition, these analyses were conducted on positive control brain (normal brain) and nontransgenic mammary tissues (Normal M.gl.). The positions of c-Src and c-Yes kinases and exogenous enolase substrate are illustrated by the arrows.

(B) Immunoprecipitation of identical tumor and control tissue protein extracts with antisera directed against PyV middle T antigen followed by immunoblot analyses with c-Src or c-Yes specific antibodies. Also included are negative control protein extracts derived from nontransgenic mammary glands (M.gl.). The broad lower band is due to crossreactive immunoglobulin band present in the immunoprecipitates.
To determine whether the activation of c-Src and c-Yes was due to its association with the PyV middle T product, tumor extracts were immunoprecipitated with a PyV middle T specific antisera. After gel electrophoresis, these immunoprecipitates were subjected to immunoblot analyses with antisera directed against either c-Src or c-Yes proteins. Because Fyn kinase activity was not significantly elevated in the PyV middle T antigen induced tumors, no comparable analyses was performed with the Fyn specific antisera. The results of these analyses revealed the presence of both the c-Src and c-Yes proteins in the PyV middle T immunoprecipitates (Fig. 5.1B). In addition to c-Src and c-Yes, a lower broad band corresponding to mouse immunoglobulin was also observed. As expected, application of this methodology to protein extracts derived from normal nontransgenic mammary epithelium failed to demonstrate the presence of either c-Yes or c-Src due to the absence of the middle T antigen. Together these results indicate that PyV middle T antigen-induced mammary tumors possess elevated c-Src and c-Yes kinase activities which occur through their association with PyV middle T antigen.

5.2.2. A Functional c-Src is Required for the Rapid Induction of Metastatic Mammary Tumors.

Although the middle T antigen-induced mammary tumors possess elevated c-Src and c-Yes tyrosine kinase activities, it is unclear to what extent activation of each of these individual tyrosine kinases contributes to the overall transformed phenotype. To determine whether c-Src is required for
PyV middle T antigen mediated tumorigenesis, mice carrying a disrupted c-src gene (Soriano et al., 1991) were interbred with the MMTV/PyV middle T antigen transgenic mice (MT#634; Guy et al., 1992b). Using this approach, a variety of different genotypes of MMTV/PyV MTAg were generated including heterozygous transgene carriers in wild type c-src (MT/+, c-src +/-), heterozygous c-src (MT/+, c-src +/−) and null c-src backgrounds (MT/+, c-src −/−) (Fig. 5.2). The genotypes of each progeny were confirmed by Southern blot hybridization with appropriate transgene and c-src specific probes. Consistent with previous observations (Guy et al., 1992a), all female transgenic progeny possessing one functional c-src allele developed multifocal mammary tumors that eventually enveloped the entire mammary epithelium by 120 days (Figure 5.3A). The onset of mammary tumor formation between transgenic mice carrying both wild type c-src alleles (n=30) or heterozygous for c-src mutation (n=33) was not significantly different. By contrast, none of the MMTV/PyV MTAg transgenic mice homozygous for the c-src mutation (n=24) developed mammary tumors within this time frame (Figure 5.3A).

To exclude the possibility that the lack of tumor development in these mice was due to alteration of transgene expression, 10 μg of total RNA isolated from the mammary glands of multiparous mice was subjected to RNase protection analyses with a probe directed to the 5' segment of the PyV middle T antigen cDNA. As shown in Figure 5.3B, the transgene-specific probe yields a 203-nucleotide protected fragment. To ensure that equal quantities of RNA were loaded, an rpl32 antisense probe directed against the mouse ribosomal protein, was also included in the hybridization reaction.
Figure 5.2. Interbreeding strategy between the MMTV/PyV middle T antigen transgenic mice (MT#634) and the c-src null mice.
Figure 5.3. c-Src is required for the induction of mammary tumors in the MMTV/PyV middle T antigen transgenic mice.

(A) Transgenic mice carrying the PyV middle T oncogene in a wild type c-src (left panel, MT#8314, 70 days of age) or null c-src (MT#7832, 140 days of age) genetic backgrounds. Note the extensive mammary tumors in all mammary glands of the MT#8314 mouse and the lack of palpable tumors in the MT#7832 mouse.

(B) RNase protection analyses using 10 μg of total mammary tissue RNA isolated from multiparous females carrying the middle T transgene in wild type, heterozygous and homozygous c-Src backgrounds. Mammary tissue extract from a c-src-/ nontransgenic animal was included as a negative control. The antisense probe used in this RNase protection analyses protects a 203-nucleotide fragment corresponding to the 5' end of the PyV middle T cDNA. To ensure that equal amounts of RNA were loaded on the gels, a rPL32 antisense probe directed against the mouse ribosomal protein L32 was also included in the hybridization reaction. The L32 probe protects a 278-nucleotide fragment as indicated by the arrow.
A.

MT (+/-); c-src (+/+)

MT (+/-); c-src (-/-)

B.

<table>
<thead>
<tr>
<th>c-src (-/-)</th>
<th>MT (+/-)</th>
<th>MT (+/-); c-src (+/-)</th>
<th>MT (+/-); c-src (-/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#8529</td>
<td>#8466</td>
<td>#8476</td>
<td>#8087</td>
</tr>
<tr>
<td>#8466</td>
<td>#8476</td>
<td>#8087</td>
<td>#8464</td>
</tr>
<tr>
<td>#7882</td>
<td>#7951</td>
<td>#7848</td>
<td>#8209</td>
</tr>
</tbody>
</table>

MT 208bp

L32 276bp
The results showed that the mammary glands of mice derived from different c-src genotypes expressed equivalent levels of transgene RNA (Fig. 5.3B). Consistent with the results of these RNase protection analyses, immunoblot analyses with PyV middle T antigen antisera revealed equivalent levels of PyV middle T protein within the mammary epithelium of these mice (data not shown). Therefore, the inability of PyV middle T antigen mice to develop tumors in a c-src null genetic background was not due to differences in transgene expression.

The histological appearance of the mammary tissue derived from MMTV/middle T antigen transgenic mice carrying at least one functional c-src allele exhibited dramatic differences in comparison to mammary tissue from transgenic mice homozygous for the disrupted c-src gene (Figure 5.4). By contrast to wild type FVB mammary glands (Figures 5.4A and 5.4B), whole-mount examination of virgin mammary tissue from female MMTV/PyV middle T antigen mice heterozygous for the c-src mutation revealed the presence of multiple mammary adenocarcinomas as early as 60 days of age (Fig. 5.4C). These analyses failed to detect comparable histological lesions in older virgin transgene carriers (100 days of age) lacking c-src function (data not shown). In older multiparous or virgin female transgenic mice homozygous for the disrupted c-src gene, focal mammary epithelial hyperplasias have been detected (Fig. 5.4E). Although these focal mammary epithelial hyperplasias can eventually envelope the entire mammary fat pad, they rarely progress to full malignancy. In fact, of the female transgenic mice lacking c-Src function that have lived to an age of 3 months or older (n=24),
Figure 5.4. Histopathology of the MMTV/PyV middle T antigen mice carrying disrupted *c-src* alleles.

A panel of photomicrographs showing the appearance of hematoxylin stained whole mount (left hand side: A, C, and E, magnification: x9 and hematoxylin/eosin stained microscopic section (right hand side: B, D, and F, magnification: x90). Virgin female FVB animal (A and B), MT (+/-); *c-src* (+/-) (MT#7698 at 75 days of age) (C and D) and MT(+/-); *c-src* (-/-) (MT#7832 at 140 days of age) (E and F) transgenic animal. Note the slender, nonbranching ducts of the wild type animal, the proliferative multilayered structures in the MT#7698 animal and the dilated complex ducts lined by a single epithelial layer in the MT#7832 animal.
only two animals have developed a focal mammary adenocarcinoma, and this occurred only after long latency (9 months of age). Consistent with these findings, the mammary epithelial hyperplasias observed in the c-src deficient background are histologically distinct from the middle T antigen-induced mammary tumors. By contrast to the proliferative multilayered epithelium observed in the PyV middle T antigen-induced mammary tumors, the hyperplasias observed in the c-Src null background are comprised of complex ducts lined by a single epithelial layer (Figure 5.4D and 5.4F).

5.2.3. Detection of PyV Middle T Associated Tyrosine Kinase Activity in the Mammary Tissue of c-Src Deficient Mice.

The epithelial hyperplasias observed in the c-Src deficient mice expressing the PyV middle T oncogene could conceivably result from activation of the PyV middle T associated c-Yes tyrosine kinase. To test this possibility, Western immunoblot (Fig. 5.5) and in vitro kinase (5.6A) assays were conducted on the mammary tissue derived from MMTV/PyV MTAg transgenic mice carrying either wild type or mutant c-src alleles with antisera directed against PyV middle T antigen, c-Src and c-Yes. Incubation of these tissue extracts with antisera against the PyV middle T oncogene indicated that an autophosphorylated band corresponding to 56 kDa PyV middle T antigen could be detected in mice heterozygous or homozygous for the c-src mutation (Fig. 5.6A). To assess whether phosphorylation of PyV middle T protein in the c-src null genetic background was due to the activation of the c-Yes tyrosine kinase, in vitro kinase assays were also conducted with antisera
Figure 5.5. Detection of the c-Src and c-Yes in MMTV/PyV middle T mammary tissues defective in c-Src function.

Western blot analysis of MMTV/PyV middle T mammary tissues defective in c-Src function were performed using specific antibodies for both the c-src and c-yes products. Mammary tissue extracts derived from multiparous female MT(+/−); c-src (+/+)(MT#8466 at 90 days of age), MT(+/-); c-src (+/-) (MT#7919 at 90 days of age) and MT(+/-); c-src (-/-) (MT#7915 at 160 days of age) were analysed. In addition, brain tissue from a normal mouse (Normal Brain) or from a nontransgenic c-src and c-yes null animal (Brain c-src -/-; Brain c-yes -/-) were used as positive and negative control for c-Src and c-Yes. Nontransgenic normal mammary tissue was also included (Normal M.gl) as a supplementary negative control.
Figure 5.6. Detection of PyV middle T associated c-Yes kinase activity in mammary tissues defective in c-Src function.

(A). Shown are, in vitro kinase activities of PyV middle T antigen, c-Src, and c-Yes in mammary tissue extracts derived from multiparous female MT(+/−); c-src (+/+)(MT#8466 at 90 days of age) and MT(+/-); c-src (+/-)(MT#7919 at 90 days of age) and MT(+/-); c-src (-/-)(MT#7915 at 160 days of age). Protein extracts were immunoprecipitated with either a rat polyclonal antisera against PyV middle T antigen, a monoclonal antibody that recognizes c-Src (Mab 327, Oncogene Sci) or an antipeptide polysera specific for c-Yes (lanes marked +). Normal mouse sera was also used as a nonspecific control (lanes marked −). In addition, these analyses were conducted on brain tissue which served as a positive control for c-Src and c-Yes and on a PyV middle T expressing 293 cell extract (MT/293) which served as a positive control for PyV middle T protein. Nontransgenic mammary tissue was also included (Normal M.g.L) which served as a negative control. The position of the PyV middle T antigen, c-Src and c-Yes are illustrated by the arrows.

(B) Immunoprecipitation of identical tumor and control tissue extracts with antibodies directed against PyV middle T antigen followed by immunoblot analyses with either c-Src or c-Yes specific antibodies. Also included are positive control protein extracts from PyV middle T transformed rat fibroblasts (MT-1).
directed against the c-Yes and c-Src proteins. As expected, *in vitro* kinase analyses with c-Src specific antibodies showed no evidence of c-Src kinase activity in mammary tissues obtained from transgenic mice lacking c-src function (Fig. 5.6A). However, comparable levels of c-Yes associated kinase activity could be detected in tissues from transgenic mice carrying either wildtype or disrupted c-src genes (Fig. 5.6A). Consistent with these observations, is the detection by Western blot analysis of c-Yes but not c-Src in mammary tissue samples derived from the MMTV/MTAg transgenic mice homozygous for the disrupted c-src allele (Fig 5.5).

To confirm that the observed PyV middle T antigen-associated kinase activity in the c-src null background was due to its association with the c-Yes tyrosine kinase, tumor extracts from transgenic mice harboring the c-src mutation were subjected to immunoprecipitation with an antibody directed against the PyV middle T antigen followed by immunoblot analyses with either a c-Yes or a c-Src specific antisera. As shown in Figure 5.6B, both c-Src and c-Yes were associated with PyV middle T antigen in mice wild type or heterozygous for the c-src mutation (Figure 5.6B). However, the PyV MTAg transgenic mice homozygous for the disrupted c-src gene, was found complexed with only the c-Yes tyrosine kinase. These observations suggest that the mammary epithelial hyperplasias observed in the MMTV/PyV MTAg transgenic mice lacking a functional c-Src is due to the activation of c-Yes tyrosine kinase by PyV middle T antigen.

It is also possible that c-Src may have substrates distinct from c-Yes that are required for cellular transformation. In this regard, analyses of the mammary gland extracts from c-Src deficient and wild type mice carrying the
transgene with antiphosphotyrosine containing antibodies has revealed no obvious differences in the pattern of tyrosine phosphorylated proteins between these tissues (Fig. 5.7). However, these analyses may not be sensitive enough to detect subtle differences in substrate specificity between c-Src and c-Yes.

5.2.4. c-Yes is dispensable for PyV middle T mediated mammary tumorigenesis.

One possible interpretation for the phenotype seen in the cross between the MMTV/PyV middle T and c-Src deficient strains is that transformation of the mammary epithelial cell by the PyV middle T oncogene requires the activity of both the MTAg/c-Src and MTAg/c-Yes complexes to transform the mammary epithelial cell. To explore this possibility further, the MMTV/middle T antigen strains were crossed with c-Yes deficient mice (Fig. 5.8). Unlike the c-Src deficient mice which suffer from osteopetrosis (Soriano, et al., 1991), the c-Yes deficient mice display no obvious abnormalities (P. Soriano, personal communication).

As shown in Figure 5.9, the onset of mammary tumor formation between mice carrying one or both wild type c-Yes alleles did not significantly differ. Interestingly, all female transgenic mice expressing the middle T transgene in the c-Yes deficient background developed multifocal mammary tumors (Fig. 5.9). Consistent with previous observations (Guy et al., 1992b), all female transgenic progeny possessing one functional c-yes allele developed
Figure 5.7. Comparison between pattern of tyrosine phosphorylated proteins in mammary tissue of MMTV/PyV middle T mice defective in c-Src function.

Antiphosphotyrosine immunoblot of mammary tissue extracts derived from multiparous female MT(+/-); c-src (+/+)(MT#8466 at 90 days of age), MT(+/-); c-src (+/-)(MT#7919 at 90 days of age) and MT(+/-); c-src (-/-)(MT#7915 at 160 days of age). Control tissue obtained from normal mammary gland of female FVB mice (Normal M.gl.), nontransgenic c-Src defective animal (M.gl. c-src-/-) and middle T antigen transformed cell line (MT-1) are also included.
Figure 5.8. Interbreeding strategy between the MMTV/PyV middle T transgenic mice (MT#634) and the c-Yes null mice.
MT#634 (+/-) \rightarrow X \rightarrow \\
Yes (-/-) \\

MT (+/-) ; c-yes (+/-) \rightarrow X \rightarrow \\
c-yes (-/-) \\

MT (+/-) ; c-yes (-/-)
Figure 5.9. Kinetics of tumor occurrence of the MMTV/PyV middle T antigen in a wild type, heterozygous and homozygous c-Yes backgrounds.

Comparison of the kinetics of tumor formation between female transgenic carriers bearing the MMTV/PyV middle T antigen wild type c-yes, MT (+/-), c-yes (+/+), heterozygous c-yes, MT(+/-), c-yes (+/-) and null c-yes backgrounds MT(+/-), c-yes (-/-). The age at which 50% of mice were found to have tumors (t50) and the number of mice examined (n) are also indicated.
multifocal mammary tumors that eventually enveloped the entire mammary epithelium by 100 days (Fig. 5.9). The onset of mammary tumor formation between transgenic mice carrying both wild type c-yes alleles (n=30) or heterozygous for the c-yes mutation (n=26) were identical (T50=58 days). By contrast, the MMTV/PyV middle T transgenic mice homozygous for the c-yes mutation (n=31) developed mammary tumors over a longer period of time (Fig. 5.9). For example, 50% of the MMTV/PyV middle T mice in a null c-Yes background have developed mammary tumors within 80 days. This delay in tumor formation also allowed the female carriers to nurse their young through one pregnancy. Despite the delayed kinetics of tumor formation, all multiparous MMTV/PyV MTAg female carriers deficient for c-Yes developed mammary tumors that enveloped the entire mammary epithelium.

The mammary tumors that arose in the c-Yes deficient mice were histologically indistinguishable from tumors observed in the original MMTV/PyV middle T strains (data not shown). To test whether the tumors arising in the c-Yes deficient strains resulted from the activation of PyV middle T associated c-Src kinase activity, in vitro kinase assays were conducted on mammary tissue derived from various genotypes with antisera specific for PyV middle T antigen, c-Src and c-Yes (Fig. 5.10). Mammary tumors derived from transgenic mice heterozygous or homozygous for the disrupted c-yes alleles possessed 68% or 33%, respectively, of the middle T antigen-associated kinase activity observed in the wild-type c-Yes background (Fig., 5.10). In vitro kinase analyses with c-Src-
Figure 5.10. Polyomavirus middle T antigen-associated c-Src kinase activity in mammary tumors of mice lacking functional c-Yes.

Tissue extracts from mammary tumors of multiparous females MT(+/-); c-yes(+/-) (MT#1907 at 90 days of age), MT(+/-); c-yes(+/-) (MT#39 at 138 days of age), MT(+/-); c-yes(-/-) (MT#119 at 150 days of age) were used for in vitro kinase assays of middle T antigen (A), c-Src (B), and c-Yes (C). Protein extracts were incubated with Glu-Glu antibody, which recognizes PyV middle T antigen, with monoclonal antibody recognizing Src (Ab.1, Oncogene Sci.), or with a monoclonal antibody recognizing Yes (3H9). Protein lysates from brain and nontransgenic mammary gland (normal M.gl.) were used as +ve and -ve controls, respectively. Normal rabbit serum was incubated with lysates from brain tissue to serve as nonspecific control (-ve). The position of enolase, c-Src, and c-Yes are indicated by arrows.
and c-Yes-specific antisera revealed comparable levels of c-Src kinase activity between wild-type and c-Yes-deficient strains (Fig., 5.10). However, no detectable c-Yes kinase activity could be observed in middle T antigen/c-Yes deficient mice (Fig., 5.10). Taken together, these findings argue that activation of the c-Yes kinase is not required for the induction of mammary tumors by the PyV middle T antigen.

5.3. Discussion

Our observations provide evidence that c-Src activity is required for the rapid induction of metastatic mammary tumors in transgenic mice expressing the PyV middle T antigen. By contrast to the rapid development of mammary tumors observed in the MMTV/PyV middle T antigen mice heterozygous for a disrupted c-src gene (Soriano et al., 1991), mice expressing PyV middle T antigen in the absence of a functional c-Src rarely develop mammary tumors. However, these mice eventually develop benign mammary epithelial hyperplasias which correlated with the activation of the PyV middle T associated c-Yes kinase. Conversely, mice expressing the middle T transgene in a c-Yes null background develop multifocal mammary tumors with 100% penetrance. These observations support the hypothesis that activation of a signal transduction pathway involving c-Src is responsible for the rapid production of metastatic mammary tumors observed in the MMTV/PyV middle T transgenic mice.

Because the PyV middle T oncogene is known to associate and activate the c-Src, c-Yes and Fyn tyrosine kinases (Courtneidge and Smith 1983,
Kornbluth et al., 1986, Cheng et al., 1988, Kypta et al. 1988), the activity of these kinases was initially assessed in the mammary tumor extracts derived from different MMTV/PyV middle T strains using in vitro kinase assays. Consistent with observations made with middle T oncogene-expressing fibroblast cell lines, association of PyV middle T antigen with c-Src and c-Yes resulted in activation of their intrinsic tyrosine kinase activities. While the Fyn tyrosine kinase is capable of associating with PyV middle T antigen, this protein-protein interaction does not result in a significant increase in its tyrosine kinase activity in fibroblasts (Cheng et al., 1988; Kypta et al., 1988) or in middle T antigen induced mammary tumors (data not shown). By contrast, the closely related hamster PyV (HaPyV) encodes a middle T product that is capable of associating and activating the Fyn tyrosine kinase but was unable to complex with the c-Src and c-Yes tyrosine kinase (Courtenedge et al., 1991). Interestingly, unlike the mouse PyV which induce a variety of epithelial tumors including mammary tumors (Berebbi et al., 1990), expression of hamster PyV middle T antigen is associated with the induction of lymphoid tumors (Courtenedge et al., 1991). It is conceivable that the tumor type induced by these viral oncogenes may be dependent on the nature of Src family member which is activated. Future experiments directed towards expressing the HaPyV middle T antigen in the mammary epithelium should allow this question to be addressed.

Direct evidence for the involvement of the c-src proto-oncogene in mammary tumorigenesis derives from results obtained from the interbreeding between the MMTV/PyV middle T antigen transgenic mice and mice encoding a disrupted c-src gene (Soriano et al., 1991). Although we
could detect expression of middle T antigen encoded protein and RNA from the mammary glands of these c-Src deficient transgenic mice, these animals rarely developed mammary tumors. By contrast, all those transgenic mice which were heterozygous for the disrupted c-src allele or carried both wild type c-src alleles, developed multifocal mammary tumors with similar kinetics. Indeed, these mice also developed metastasis to the lung with high frequency (95%). Although the MMTV/PyV middle T antigen mice lacking c-Src function rarely develop mammary tumors, mammary epithelial hyperplasias were often observed in these animals (Fig. 5.4E and 5.4F). It is conceivable that these hyperplasias are the result of activation of c-Yes by PyV middle T antigen since elevated levels of c-Yes kinase activity can be detected in these tissues. However, given the infrequent occurrence of mammary tumors in these mice, the additional activation of c-Src appears to be required for the mammary cell to acquire the full malignant phenotype.

Further evidence implicating the PyV MTAg/c-Src complex in mammary tumorigenesis derives from observations from the interbreeding of the MMTV/middle T antigen strains with the c-Yes deficient mice. Unlike the MMTV MTAg/c-Src deficient mice, transgenic mice expressing the middle T oncogene in a c-Yes null background develop multifocal mammary tumors with 100% penetrance (Fig. 5.9). The inability of the PyV MTAg/c-Yes complex to efficiently transform the mammary epithelium in the absence of c-Src does not appear to be the result of an overall lower level of PyV MTAg-associated kinase activity since the levels of PyV MTAg-associated kinase are comparable between the c-Src and c-Yes deficient strains. One possible explanation for these observations, is that the MTAg/c-Src complex may have
substrates that are distinct from the MTAg/c-Yes complex which are required for cellular transformation. In this regard, analyses of the mammary gland extracts from c-Src deficient, c-Yes deficient and wild type mice carrying the transgene with antiphosphotyrosine specific antibodies has revealed no obvious differences in the pattern of phosphorylated proteins between these tissues (Fig.5.7). However, these analyses may not be sensitive enough to detect subtle differences in substrate specificity between c-Src and c-Yes.

Another possible explanation for these findings, is that c-Src and c-Yes are expressed in different cell types in the mammary gland, and that tumor precursor cells only express c-Src. However, the immunoprecipitation and immunoblot analyses with PyV MTAg specific antibodies and either c-Src or c-Yes specific antibodies revealed that middle T antigen is complexed with both c-Src and c-Yes. Because the expression of middle T antigen is driven by the MMTV/LTR and this enhancer is transcriptionally active in identical cell types, these observations argue that c-Src and c-Yes are coexpressed in the mammary tumor cell. Alternatively, it is also possible that inactivation of c-Src indirectly affects tumor formation by affecting secondary factors involved in tumor progression. Indeed, the c-src deficient mice develop osteopetrosis and are runted (Soriano et al., 1991). However, the induction of other tumors by PyV middle T antigen is not affected by the disruption of c-Src in these mice (Thomas et al., 1993).

Another cell type that is exquisitely sensitive to transformation by middle T antigen is the endothelial cell (Bautch et al., 1987; Williams et al., 1988). By contrast to the mammary epithelial cell, transformation of the endothelial cell or establish fibroblasts by PyV middle T antigen does not
require functional c-Src (Thomas et al., 1993). However, endothelial expression of middle T antigen in a c-Yes deficient background resulted in reduction of the number of endothelial tumors which arose after a longer latency period (Kiefer et al., 1994). Conversely, in certain PyV transformed rat cell lines, inducible expression of an antisense c-src construct results in the reduction of the tumorigenic properties of these lines (Amini et al., 1986). Hence, activation of closely related Src-family tyrosine kinases may have dramatically different outcomes in different cell types.

Consistent with these observations, there are several recent reports demonstrating elevated levels of c-Src tyrosine kinase activity in a large proportion of primary human breast cancers (Jacobs and Rubsamen, 1983; Rosen et al., 1986; Ottenhoff-Kalff et al., 1992). Because equivalent levels of c-Src protein were detected in matched normal and tumor tissues, the elevated c-Src tyrosine kinase activity observed is likely due to qualitative rather than quantitative changes in the regulation of c-Src activity (Rosen et al., 1986). In fact, we have recently detected elevated c-Src and c-Yes kinase activity in mammary tumors derived from mice carrying an MMTV/unactivated neu transgene (Guy et al., 1992b; Muthuswamy et al., 1994). It is conceivable that like the PyV middle T oncoprotein, Neu induced mammary tumorigenesis may also requires the function of these Src family members. Indeed, ligand dependant activation of other receptor tyrosine kinases like the platelet derived growth factor receptor (PDGFR) (Kypa et al., 1990) or the colony stimulating factor 1 receptor (CSF-1R) (Courtneidge et al., 1993) results in the activation of c-Src, c-Yes and Fyn tyrosine kinase activities. Microinjection of dominant negative mutants of c-Src into cells can effectively ablate PDGF
mediated mitogenesis (Twamley-Stein et al., 1993). Future crosses between the MMTV/unactivated Neu mice and the c-Src deficient mice should allow us to determine whether or not c-Src is required for Neu mediated mammary tumorigenesis.

While these experiments have focused on the role of c-Src in PyV middle T antigen induced tumorigenesis, this study may have general implications in understanding how oncogene products individually participate in a signal transduction pathway in vivo. For example, it should be possible to assess the relative contribution of other components of a tyrosine kinase signal transduction pathway to the tumor phenotype by interbreeding these mice to other strains deficient in genes thought to be involved in signalling cellular proliferation. Application of this genetic approach to other mitogenic signal transduction pathways, may provide important insights into understanding how oncogene products collaborate in mammary tumorigenesis.
CHAPTER 6

CONCLUSION

The aim of this thesis is to understand the role and mechanism of action of tyrosine kinases in mammary tumorigenesis. Because very little information is available on the nature of the genetic alterations involved in the development or progression of primary breast adenocarcinomas, I was interested in creating useful animal models to study its genetic requirements. Consequently, I have used the transgenic mouse as a tool to directly assess the consequences of expression of either the Neu or polyomavirus middle T antigen-associated tyrosine kinases in the mammary epithelium.

To directly assess the effect of mammary gland-specific expression of the neu proto-oncogene, transgenic mice carrying unactivated neu under the transcriptional control of the mouse mammary tumor virus promoter/enhancer were established. By contrast to the rapid tumor progression observed in several transgenic strains carrying the activated neu transgene (Muller et al., 1988; Bouchard et al., 1989), expression of unactivated neu in the mammary epithelium resulted in the development of metastatic focal mammary tumors after long latency. The majority of the mammary tumors analyzed expressed elevated levels of neu-encoded mRNA and protein. Overexpression of neu in mammary tumors was also associated with elevated Neu intrinsic tyrosine kinase activity by comparison to the adjacent normal mammary epithelium. The unexpected finding that many
of the older tumor-bearing neu transgenic animals developed pulmonary metastases may have important clinical implications. These observations provide the first direct evidence that expression of the proto-oncogenic form of neu results in a heritable development of metastatic mammary tumors. In addition, these data also imply that the deregulated expression of tyrosine kinases are critical events in the initiation and progression of human breast cancer.

Another potent tyrosine kinase activity that has been implicated in the genesis of murine mammary tumors is that associated with polyomavirus middle T antigen. Analysis of the transforming properties of the PyV middle T oncogene in the mammary epithelium provides important insight into the process of malignant progression. By contrast to most transgenic strains carrying activated oncogenes, expression of MMTV/PyV middle T antigen resulted in the widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas that metastasize to the lung with high frequency. The potent transforming activity of PyV middle T antigen in the mammary epithelium can, in part, be attributed to its ability to associate with and activate a number of Src family tyrosine kinases (c-Src, c-Yes, and Fyn). In order to assess the role of individual c-Src family tyrosine kinases in PyV middle T antigen-induced mammary tumorigenesis, I have crossed transgenic mice carrying the MMTV/PyV middle T antigen fusion gene with mice bearing a disrupted c-src or c-yes proto-oncogenes. In contrast to the rapid tumor progression seen in the original MMTV/PyV middle T antigen strains, mice expressing the transgene in the absence of functional c-Src rarely developed mammary
tumors. After long latency, these mice did eventually develop abnormal hyperplastic mammary tissue. This growth disturbance was correlated with elevated expression of the PyV middle T antigen and the activation of the PyV middle T antigen-associated c-Yes tyrosine kinase. However, transgenic mice expressing the PyV middle T antigen in the mammary epithelium of wild-type or Yes-deficient mice developed multifocal mammary tumors with comparable kinetics. Taken together, these findings suggest that c-Src tyrosine kinase activity is required for PyV middle T antigen induced mammary tumorigenesis whereas c-Yes is dispensable.

Consistent with these results is the recent observation that activation of c-Src may play a role in the genesis of human cancer. Several studies have implicated c-Src in the progression of colon cancer. Measurement of the in vitro protein tyrosine kinase activity of c-Src in polyps and colon carcinomas revealed that these tissues possessed elevated levels of c-Src activity by comparison to adjacent normal mucosa (Bolen et al., 1987; Cartwright et al., 1989; 1990). In addition, because c-Src activation was detected in polyps before the development of carcinoma, it was predicted to be an early event in tumorigenesis (Cartwright et al., 1990).

Further evidence implicating c-Src tyrosine kinase activity in tumorigenesis derives from the observation that elevated levels of c-Src tyrosine kinase activity can be detected in a large proportion of primary human breast cancers (Jacobs and Rubsamen, 1983; Rosen et al., 1986; Ottenhoff-Kalf et al., 1992; Luttrell et al., 1994). In one study, more than 70% of the PTK activity detected in malignant breast tissue was due to the activation of the c-src oncogene product (Ottenhoff-Kalf et al., 1992). Indeed,
constitutive activation of these signaling pathways is apparent in many malignancies. Interestingly, the product of a newly identified gene called EMS-1, has been shown to be frequently amplified and overexpressed in human breast cancer (Schuuring et al., 1992). This gene product, p85, is 85% homologous to a chicken protein that was recently identified as a substrate for the c-src oncogene (Wu et al., 1991; Schuuring et al., 1993). Moreover, the EMS-1 protein has been localized at the cell-substratum contact sites and might therefore, contribute to the invasive potential of these tumor cells (Schuuring et al., 1993).

Consistent with these clinical studies, mammary gland specific expression of PyV middle T antigen in transgenic mice results in the rapid induction of mammary tumors due to the activation of the c-Src tyrosine kinase pathway (Guy et al., 1992a). Further evidence supporting the role of c-Src in PyV middle T antigen-induced tumorigenesis derives from the results of the interbreeding of the MMTV/PyV MTAg mice with mice bearing a germline mutation in c-Src (Soriano et al., 1991). Because transgenic mice expressing PyV middle T antigen in the absence of c-Src rarely developed mammary tumors, activation of this pathway is critical for tumor formation (Guy et al., 1994).

In addition to the PyV middle T antigen, overexpression of neu in the mammary epithelium of transgenic mice also led to a dramatic elevation of the c-Src kinase activity in mammary tumors (Muthuswamy et al., 1994). Moreover, these studies demonstrated that the increased of c-Src tyrosine kinase activity observed in Neu-induced mammary tumors was not due to an increase in the levels of c-Src but rather was a result of the elevation of its
specific activity (Luttrell et al., 1994; Muthuswamy et al., 1994). Interestingly, both studies showed that the activation of c-Src was also correlated with its ability to complex tyrosine-phosphorylated Neu in vitro and in vivo (Luttrell et al., 1994; Muthuswamy et al., 1994).

The contention that Neu may signal cell proliferation through activation of c-Src has been supported by studies of other receptor tyrosine kinases. For example the platelet-derived growth factor receptor (PDGFR), the epidermal growth factor receptor (EGFR) and the colony stimulating factor-1 receptor (CSF-1R) are all able to associate with and/or activate different members of the Src family kinase (Kypta et al., 1990; Courtneidge et al., 1993; Luttrell et al., 1994). Thus, interaction of Src family members with receptor tyrosine kinases may be a general feature of signaling mediated through these types of growth factor receptors.

Signaling mediated by receptor tyrosine kinases, such as the epidermal growth factor receptor or the Neu receptor, requires receptor autophosphorylation on tyrosine (Ulrich and Schlessinger, 1990). These phosphotyrosine residues serve as unique binding sites for protein that contain SRC homology 2 domains. Such domains are found in a number of proteins involved in intracellular signaling including p85, the noncatalytic subunit of PI3'kinase, GAP, the GTPase-activating protein of Ras, phospholipase Cγ, and protein phosphatase 1D (Arteaga et al., 1991; Fazioli et al., 1991; Segatto et al., 1992; Vogel et al., 1993) and are thought to be involved in signal transduction from activated receptor tyrosine kinases such as Neu.

The transgenic mice expressing MMTV/PyV middle T antigen and MMTV/unactivated neu constructs possess many similarities. Indeed, both
transgenic models develop metastatic mammary tumors. The similarity in phenotypes may reflect the ability for PyV middle T antigen and Neu to signal cell proliferation and metastasis through common pathways. For example, both PyV middle T antigen and Neu activate the c-Src (Courtneidge et al., 1983; Bolen et al., 1984; Luttrell et al., 1994; Muthuswamy et al., 1994), Shc (Segatto et al., 1992; Dilworth et al., 1994), and the PI3'kinase (Withman et al., 1985; Courtneidge et al., 1987; Cantley et al., 1991; Peles et al., 1992) pathways. At least in the case of PyV middle T associated tyrosine kinases, genes encoding various members of the protease family appear to be additional potential downstream targets (Montesano et al., 1990). In fact, many of the promoter regions of these proteases contain binding sites for transcription factors such as Ets, Fos, and Jun whose activity can be stimulated through non-nuclear oncoproteins such as PyV middle T antigen (Wasylyk et al., 1991). In this regard, it is interesting to note that the neu and PyV middle T antigen induced tumors overexpress the ets related transcription factor, PEA3 (Xin et al., 1992; Trimble et al., 1993). In fact, expression of activated neu results in the transcriptional activation of a reporter construct bearing PEA3 binding sites (Muthuswamy and Muller, unpublished information). Determination of whether activation of these transcription factors through the action of the neu kinase is responsible for the induction of metastatic disease awaits further analysis.

Future directions

Although it is clear from my results that the activation of the c-Src tyrosine kinase is required for the induction of mammary tumors in
MMTV/PyV middle T antigen transgenic mice, its possible role in Neu-induced mammary tumorigenesis still remains to be established. In order to directly demonstrate a requirement for c-Src in Neu-induced tumorigenesis, it will be necessary to cross the MMTV/unactivated neu mice with mice bearing germline mutations in c-src and c-yes. These studies should provide important insight into the role of c-Src and c-Yes in mammary tumorigenesis.

Another important issue that remains to be addressed is the nature of the downstream targets of PyV middle T antigen and Neu in the mammary epithelium. Stimulation of Neu kinase activity is known to result in the downstream activation of a number of cellular enzymatic activities. For example, neu shares with other growth factor receptors the ability to induce both a rapid increase in the concentration of intracellular calcium and an elevated rate of hydrolysis of phosphoinositides (Pandiella et al., 1989). And like other PTKs, neu is likely capable of binding a number of signal transduction molecules through SH-2 phosphotyrosine interaction (Peles et al., 1992). These events have associated the coupling of neu with a phosphoinositide-specific phospholipase. Thus, activated neu appears to be constitutively coupled to the phosphoinositide-specific phospholipase C-γ (PLC-γ) which is involved in the generation of phospholipid secondary messengers (Peles et al., 1991;1992). Two different studies have also shown that the c-erbB-2 receptor phosphorylate PLC-γ (DiFiore et al., 1990; Fazioli et al., 1991). In addition, Arteaga et al. (1991) have recently demonstrated the presence of elevated content of the tyrosine kinase substrate phospholipase-Cγ1 in primary human breast carcinomas. Taken together these data suggest that phosphorylation of PLC-γ is an important event in neu-mediated
signaling and suggest that transformation by the neu/c-erbB-2 receptor involves tyrosine phosphorylation and activation of PLC-γ.

Another signaling molecule that undergoes tyrosine phosphorylation following ligand binding to chimeric Neu protein is the GTPase-activating protein of ras (Fazioli et al., 1991) and phosphatidylinositol (PI)'3 kinase (review by Cantley et al., 1991; Peles et al., 1992). The possibility that PI-3 kinase is an effector of the Neu receptor was raised by the observation that the homologous receptor for EGF is coupled to this lipid kinase (Bjorge et al. 1990). More recently, Peles et al. (1992) have demonstrated that indeed, PI-3 kinase is a physiological substrate of the Neu receptor. However, the function of this lipid kinase in cell regulation still remains unclear. Interestingly, the PI3'kinase is also activated by its association with the PyV middle T antigen. In fact, mutants of PyV middle T antigen that are unable to bind to PI3'kinase fail to induce tumors in vivo (Talmage et al., 1989). However, because these studies were conducted with virus, it is unclear whether the transformation defect observed was due to a direct effect of PI3'kinase. Further studies with transgenic mice expressing this mutant middle T in the mammary epithelium should allow this to be addressed.

Beside these enzymatic activities, a 56 kDa phosphotyrosine containing protein, which copurifies with a phosphatidylinositol 4'kinase activity, is also transiently associated with kinase active Neu molecule (Scott et al., 1991). Similarly, a number of phosphotyrosine-containing proteins, including proteins of 185 kDa and 56 kDa, were specifically detected in tumor tissue derived from the MMTV/unactivated neu and middle T antigen transgenic mice but not from the adjacent normal tissue (see Fig. 3.7). Together, these
observations suggest that activation of enzymes such as PLC-γ, PI3'kinase, and 56 kDa protein might be important element in neu signal transduction.

Another protein that is tyrosine phosphorylated by Neu and PyV middle T antigen is Shc (Dilworth et al., 1994). The importance of the interaction of PyV middle T antigen with Shc has recently been demonstrated by the observation that conversion of tyrosine 250 to a phenylalanine residue disrupts Shc/PyV middle T complex and results in transformation (Dilworth et al., 1994). Whether Shc function is required for induction of mammary tumors by these tyrosine kinases awaits further investigation.

Ultimately, the downstream targets of these receptor tyrosine kinases are thought to be nuclear transcription factors. In this regard, it is interesting to note that overexpression of the c-ets related transcription factor PEA3 (Xin et al., 1991) has been observed in Neu and PyV middle T antigen-induced mammary tumors but not in the adjacent normal mammary epithelium (Trimble, et al., 1993). Many of the genes implicated in metastasis such as the metalloproteinases are known to possess PEA3 responsive elements in their promoter regions (Wasylyk et al., 1991; Nevlov et al., 1991). Furthermore, transcription from these promoters is activated through a number of non-nuclear oncoproteins including members of the tyrosine kinase family (Wasylyk et al., 1990). Whether the elevated levels of PEA3 observed in the MMTV/unactivated neu and MMTV/PyV middle T antigen tumors is directly involved in tumor progression awaits further analysis.

Although activation of PEA3, PI3’ kinase, Shc, 56 kDa proteins may be involved in mammary tumorigenesis in MMTV/unactivated neu and MMTV/PyV middle T antigen carriers, it is also possible that other cellular
genes cooperate with neu in multistep carcinogenesis. By this approach, known or novel cellular oncogene that collaborates with neu in mammary tumor progression might be uncovered.

In summary, the analyses of transgenic mice bearing the Neu and PyV middle T associated tyrosine kinases has lead to the concept that certain proteins, involved in proliferative signal transduction, function in a tissue specific manner. The results of the interbreeding of the MMTV/PyV middle T transgenics with the c-Src and c-Yes deficient mice suggest that c-Src is the primary target utilized by PyV middle T antigen in mammary tissue. By contrast to these observations, c-Src is dispensable for PyV middle T induced transformation of endothelial cells (Thomas et al., 1993). However, in the endothelial cell, c-Yes appears to be required for PyV middle T mediated transformation. These observations suggest that relative contribution of certain signaling molecules to tumorigenesis may be highly dependent on the tissue context. Although the molecular basis of this phenomena remains to be elucidated, it is conceivable that molecules such as c-Src may have specific substrates in the mammary gland that are not shared by the closely related c-Yes kinase. Given the potential therapeutic importance of such substrates, it will be critical to identify these proteins and elucidate their mechanism of action.
Chapter 7

REFERENCES


Bignami, M., Rosa, S., Falcone, G., Tato, F., Katoh, F., and Yamasaki, H. 1988b. Specific viral oncogenes cause differential effects on cell-to-cell communication, relevant to the suppression of the transformed phenotype by normal cells. Mol. Carcinogenesis 1:67-75


Hudziak, R.M., Schlessinger, J., and Ullrich, A. 1987. Increased expression of the putative growth factor receptor p185/HER2 causes transformation


Markland, W., and Smith, A.E. 1987. Mapping of the amino-terminal half of polyomavirus middle T antigen indicates that this region is the binding domain for pp60 c-src. J. Virol. 61:285-292.


Yarden, Y. 1990. Agonistic antibodies stimulate the kinase encoded by the neu proto-oncogene in living cells but the oncogenic mutant is constitutively active. Proc. Natl. Acad. Sci. USA 87:2569-2573.


