SHC FUNCTIONS IN THE MOUSE MAMMARY GLAND

SHC FUNCTIONS IN THE DEVELOPMENT AND TRANSFORMATION OF THE MOUSE MAMMARY GLAND

By

VALERIE L. BLACKMORE, B.Sc. (Honours)

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AUTHOR: Valerie L. Blackmore, B.Sc. (Honours) (University of Guelph)

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Abstract

The adapter protein Shc is a ubiquitously expressed Src homology 2 (SH2) domain protein implicated in the transmission of activation signals to Ras. Shc proteins become phosphorylated on tyrosine in cells stimulated with a variety of growth factors and in v-*src* transformed cells and are able to transform fibroblasts and differentiate PC12 cells in a Ras-dependent fashion. To assess the transforming ability of Shc in the mouse mammary gland, I generated transgenic mice harbouring the $p52^{shc}$ cDNA under the transcriptional control of the mouse mammary tumor virus long terminal repeat (MMTV LTR). While $p52^{shc}$ expression was correlated with multiple enlarged terminal end buds in virgin mouse mammary glands, multiparous mice developed mammary hyperplasias and mammary carcinomas. The frequency, latency and focal nature with which these tumors arose suggests that additional events are necessary to induce malignant conversion of primary mammary epithelial cells. To directly test the role of Shc in established mammary tumor models, I have generated two strains of bigenic mice. When Shc was overexpressed with NDL1-2, a constitutively activated form of the Neu receptor tyrosine kinase, latency of tumor onset was decreased over that of parental MMTV/NDL1-2 mice. Polyomavirus middle T antigen (PyV MT) mutants with a functionally inactive Shc binding site (MT Y250F) are debilitated in mammary tumor formation compared to wild-type PyV MT transgenic animals. Concurrent overexpression of Shc with MT Y250F accelerated tumor kinetics and increased the propensity for metastasis to the lungs of bigenic animals.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

Shc	src homology and collagen
MMTV LTR	Mouse Mammary Tumor Virus Long Terminal Repeat
PyV MT	Polyomavirus Middle T antigen
RTK	receptor tyrosine kinase
WAP	whey acidic protein
HAN	hyperplasstic alveolar nodule
SH2	src homology 2
CH	collagen homology
PTB	phosphotyrosine binding domain
Ab	antibody
MAPK	mitogen-activated protein kinase
NDL	neu deletion
P-TYR	phosphotyrosine
IP	immunoprecipitation
WT	wild type

Contribution of Others

Some of the work described herein was generated with the help of others. In particular I would like to list the following contributions: Some of the MMTV/Shc transgenic strains were generated by Peter Siegel. Figure 1.1, Subgross morphology of the developing mammary gland, was generated by Dr. Marc Webster. Most of the transgenic genotyping was performed by Monica Graham.

Chapter 1

Introduction

Cancer is a cellular malignancy that develops from a loss of normal controls and results in unregulated growth, lack of differentiation and the ability to invade local tissues and metastasize. The condition can develop in any tissue of any organ at any age. At any point in tumor growth, cells may be in cell cycle, resting, or may move between these two phases. Small tumors have a greater percentage of cells in cycle and thus a greater potential for proliferation. Large tumors have fewer cells in cycle and a much lower proliferative activity. A subset of nonproliferating cells accounts for tumor bulk (reviewed in Cotran *et al.*, 1994). Because cancer is a disorder of cell growth and behaviour, its ultimate cause must be defined at the cellular and subcellular levels.

This introduction will briefly focus on some of the molecular signaling events involved in mammary carcinogenesis as well as normal mammary gland development. This review will introduce the recently characterized gene *shc* which encodes a cytoplasmic adaptor molecule involved in receptor tyrosine kinase (RTK) signaling (Pelicci *et al.*, 1992). The potential for modulation of RTK signaling by Shc will be discussed.

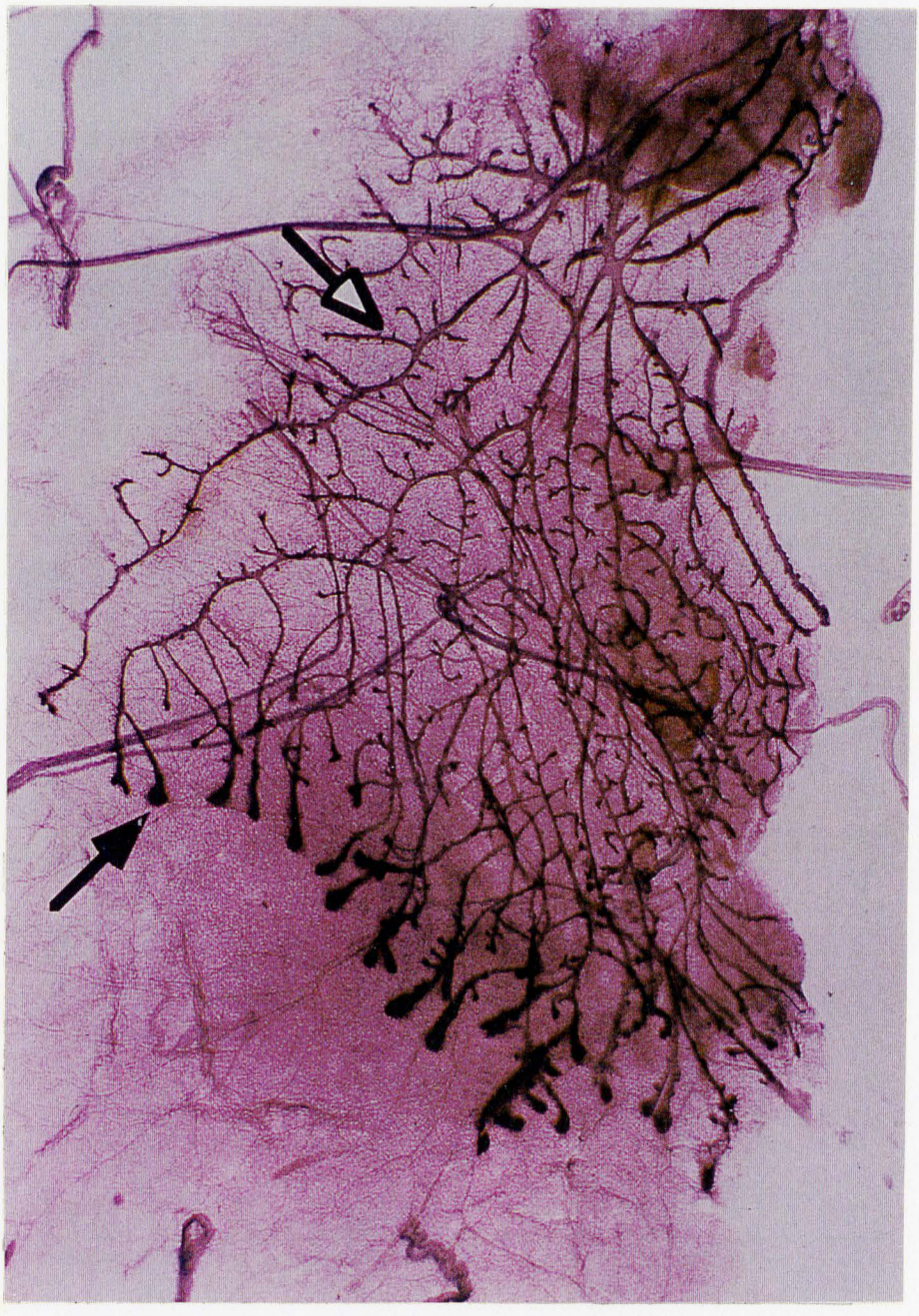
1.1 Normal mammary gland biology

The virgin mouse mammary gland is largely a ductal structure composed of one to two layers of epithelial cells. The mammary gland develops as five symmetrical buds from the milk line. At four to five weeks of age, the ducts have branched and form lobular structures. During maturation in the female, the ducts extend into the fat pad and reach the end of the fat pad between 6 to 12 weeks of age, depending upon the strain (Figure 1.1). There is little development within the female mammary gland beyond this point until pregnancy. Little initial growth occurs in the male and usually does not extend beyond the number four fat pad.

During pregnancy, the lobular units proliferate and differentiate into lobuloalveolar units containing milk-synthesizing cells. Lactating breast is composed largely of epithelium, which secretes milk proteins. TGF-α is normally expressed in human and mouse mammary glands (Liscia *et al.*, 1990) and is capable of causing lobuloalveolar development of the gland in organ culture (Vonderharr, 1988) and may stimulate epithelial proliferation in mice (Silberstein and Daniels, 1987). After weaning, most of the secretory epithelium undergoes apoptosis. The gland regresses in an orderly, physiological process, to fat cells surrounding a simple branched tree with characteristic spiculated ends (Schedin *et al.*, 1996). The present model suggests that mammary involution and epithelial cell death are initiated by lactogenic hormone ablation due to weaning (Schedin *et al.*, 1996). A failure to undergo programmed cell death may contribute to a neoplastic state (Raff, 1992).

Figure 1.1 Subgross morphology of the developing mouse mammary gland

Photomicrograph of a hematoxylin and eosin stained wholemount preparation from a four week old normal mouse mammary gland. Secondary and tertiary ductal branching is indicated by an open arrow. Epithelial end buds are indicated with a closed arrow. Magnification, 2.5X.



Normal development and maintenance of size is dependent upon a balance between proliferation and apoptosis. Involution of secretory epithelium following pregnancy and lactation is the most dramatic role of apoptosis in mammary gland development. Northern blot analyses confirm that expression of specific genes during mammary gland involution is very regulated and has been shown to involve such genes as sulfated glycoprotein-2 (SGP-2), p53, c-myc, and TGF-α-1 (Evan *et al.*, 1992).

Mammary gland involution involves tissue remodeling and massive epithelial cell death. Because mammary gland development in all mammalian species is remarkably similar, the mouse mammary gland provides an ideal model for which to study development and malignant progression of human mammary epithelial tissue. Both systemic hormones and factors mediated by epithelial and stromal interactions control the structure of the mammary gland (reviewed by Borellini *et al.*, 1989). Disruption of the normal glandular architecture can occur by one or a number of factors. During post-lactational involution the mammary gland is subjected to extensive tissue reconstruction. The cellular composition and structure of the organ change drastically according to the hormonally-defined functional state.

The process of involution involves the programmed cell death by apoptosis of the mass of secretory epithelium with associated (i) digestion and resorption of residual milk proteins, (ii) phagocytosis of the apoptotic residue of epithelial cells and (iii) coordinated degradation and reconstruction of the basement membrane in order to preserve the structural integrity of the gland (Strange *et al.*, 1992). The molecular mechanisms responsible for reorganization and maintenance are not clear.

1.2 Cancer

The prevailing view of cancer is that it is a disease that arises from a number of mutations in critical growth regulating genes. Normal growth exists as a balance between the progression of cells through their cell cycle and programmed cell death known as apoptosis. Normal development and maintenance of size of tissues depends upon the balance between cellular proliferation and cellular death. Cancer arises when there is a disruption of this balance and cells progress through the cell cycle and divide without normal control. Evidence such as aberrant chromosomes in cancerous cells (Yunis, 1996) and an apparent correlation between susceptibility to cancer and an impaired ability of cells to repair damage to their DNA (Hanawalt and Sarasin, 1986) argues that cancer is a genetic disease. Investigation of the genes affected by this damage has taught us about the elaborate mechanism that controls the proliferation and differentiation of normal cells.

Two types of genes control the proliferation of a cell. Proto-oncogenes function in a positive manner to promote growth while tumor suppressor genes provide negative growth modulation or induce apoptosis. The discovery of oncogenes removed any doubt that cancer is a genetic disease (Cantley, 1991). Observations made from RNA and DNA tumor viruses led to the belief that oncogenes are genes which are capable of inducing and maintaining cell transformation (Varmus *et al.*, 1984). The majority of these viral oncogenes have cellular homologues, referred to as proto-oncogenes.

It has now been well established that carcinogenesis occurs via an accumulation of discrete and irreversible occurrences which convert a normal cell, through a series of

pre-malignant phenotypes to a malignant state (Bishop, 1991). Advances in molecular biology support the idea that these changes all occur at the genetic level. The genetic damage seen in cancer can be one of two types. Dominant mutations are able to transform regardless of normal cellular counterparts and usually target proto-oncogenes, often resulting in a gain of function by altering gene expression or altering the gene product itself. The targets of recessive mutations are referred to as tumor suppressors and result in a loss of function. Inactivation of tumor suppressor genes may play an important role in the induction of many tumor types, including breast carcinomas (Marshall, 1991). Functional inactivation of the retinoblastoma (*Rb-1)* gene, p53 (Tang *et al.*, 1988; Malkin *et al.*, 1990) or BRCA 1 (Bishop *et al.*, 1993) tumor suppressor genes are pivotal events in tumor progression. Families with inherited deletions or mutations of these genes have an increased risk of breast and other cancers (Malkin *et al.*, 1990; Weinberg *et al.*, 1990).

The discovery that a large number and variety of human cancers contained point mutations in the *ras* proto-oncogene led to the belief that mutation of DNA was at the heart of tumor formation (Barbacid, 1987). Virtually all forms of genetic damage found in human cancers have occurred spontaneously in cultured cells at detectable frequencies (reviewed in Meuth, 1990).

1.3 Mammary tumorigenesis

Breast cancer is the most common type of cancer in women and is the greatest cause of years of life lost by Canadian women (Canadian Cancer Society, 1995). One in nine North American women will develop breast cancer over a lifetime of 85 years

(American Cancer Society, 1994). Given the high incidence of this disease, there is a need to understand the mechanisms of disease progression in order to facilitate prevention and provide therapeutics.

Research has resulted in the discovery of a variety of cellular genes with altered and/or amplified products that have been implicated as factors in mammary carcinogenesis. For example, c-ErbB is amplified in 6 to 32% of breast cancer samples (Slamon *et al.*, 1987). Another family member, c-ErbB-2 is also amplified or overexpressed in approximately 30% of human breast tumors (Coussens *et al.*, 1985; King, 1985; Slamon *et al.*, 1987). Other proteins with an involvement in mammary carcinogenesis include growth factors such as TGF-α, IGFI, IGFII, PDGF, Wnt-1, *int*-2, αFGF, βFGF and FGF-5, growth factor receptors IGFR-I and IGFR-II, non-receptor tyrosine kinases *src*, Ras, and the transcription factor *myc* as well as cyclins and cyclin-dependent kinases (for review by Dickson *et al.*, 1991). These proteins are all examples of activation of positive regulators. Inactivation of negative growth regulation genes is typically seen as loss of heterozygosity (LOH) of DNA in tumors. LOH of p53 at 17p (Donehower *et al.*, 1992), BRCA 1 at 17q (Miki *et al.*, 1994) and RB at 13q (T'Ang *et al.*, 1988) are by far the most extensively characterized.

In humans, pregnancy and lactation are associated with protection against developing breast cancer but the mechanism is not well understood (Harris, *et al.*, 1992). The protective effect appears to be related to terminal differentiation and apoptotic cell death of mammary epithelium (Andres *et al.*, 1991). It has been suggested that apoptosis following pregnancy and lactation is protective, in part, because it removes the portion of

the mammary epithelium that is at risk of neoplastic development which is the proliferating and differentiating component. Re-established gland during the next pregnancy is deplete of cells carrying or susceptible to the accumulation of tumorigenic mutations. In support of this, it appears as though human mammary epithelium of nulliparous females which has not undergone involution, is at a greater risk of developing breast cancer (Harris *et al.*, 1992).

Prolonged cell survival is characteristic of cells which have failed to undergo apoptosis and of cells that comprise a premalignant mouse mammary tumor lesion associated with increased tumor risk. Hyperplastic alveolar nodules (HAN) resemble mid-pregnancy epithelium but fail to regress following lactation (Andres *et al.*, 1991; Morris and Cardiff, 1987).

1.4 Transgenic mouse models of mammary tumorigenesis

Understanding the events underlying tumorigenesis has been tremendously enhanced by the development of several *in vitro* and *in vivo* model systems. Advances in molecular biology make it possible to detect structural alterations and expression of oncogenes in human breast cancer tissue. While this work has contributed to our body of knowledge, epigenetic factors such as hormonal conditions, adjacent tissue, extracellular matrix and blood/lymphatic effects can only be addressed *in vivo*. To overcome these limitations, many researchers have turned to the transgenic mouse as an experimental animal model system to determine tissue-specific effects of oncogenes. The transgenic

mouse mammary tumor model has emerged as one of the best systems for studying the mechanism of activity of various oncogenes in the mammary epithelium.

Most transgenic studies involve expression of a heterologous transgene within specific tissues. Direction of oncogene expression can be accomplished by a variety of mammary gland specific promoter elements fused to the transgene. The two most widely used promoter elements are the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) promoter/enhancer and the whey acidic protein (WAP) (reviewed in Muller *et al.*, 1991). Both promoters enable high levels of transcription primarily in the mammary epithelium. However, the temporal patterns of their expression and affected cell type differ.

MMTV is steroid sensitive and confers high levels of transcription to the mammary epithelium throughout all stages of mammary gland development (Pattengale *et al.*, 1989) with the highest levels being expressed at parturition (Hagar, 1988). Expression can also be detected in the Harderian gland, seminal vesicles, epididymis, salivary gland and prostate gland (Pattengale *et al.*, 1989).

WAP promoter-directed expression is restricted to later stages of pregnancy when endogenous WAP is expressed and allows for the study of transgenes in a well-differentiated state of the mammary gland (Andres *et al.*, 1988).

1.5 Multistep models of mammary tumorigenesis

The mouse mammary tumor system has traditionally been used as an experimental system for studying the biology of multistep neoplastic progression (Morris

and Cardiff, 1987). The prevailing view of mammary tumorigenesis is that a mammary cell undergoes a series of transformations (reviewed by Cardiff, 1984). The first stage is nodulogenesis, a transformation of normal cells to focal, nodular cells described as hyperplastic alveolar nodules (HAN) which contain immortal cells. These nodules are considered premalignant as they are not able to grow outside of the mammary fat pad nor are they able to metastasize. Progression to a tumor state involves the development of a malignant subset of cells from the preneoplastic hyperplasia (Schedin *et al.*, 1996). The exponential relationship of cancer incidence to age suggests that multiple events are required for tumorigenesis. Molecular analysis of human cancers, which are typically clonal, show multiple genetic lesions within a single tumor including chromosomal translocations, gene amplification , point mutations, constitutive activation of oncogenes and loss of growth suppressor genes. Statistical evidence suggests that 5 to 6 steps are required to generate a diagnosable tumor (Peto *et al.*, 1975).

Normal cells have multiple independent mechanisms to regulate growth and differentiation. Several separate events seem to be necessary to override these mechanisms and induce transformation (reviewed by Hunter, 1991). The mouse skin carcinoma model supports a multistep process (reviewed by Balmain and Brown, 1988). Application of a noncarcinogenic dose of an initiating carcinogen to mouse skin fails to cause a carcinoma until followed by a tumor promoter, which alone, has no effect. Molecular analysis shows that H-*ras* gene was mutated but was not sufficient to allow the cell to progress to a tumor until the tumor promoter treatment (Balmain *et al.*, 1984).

A role for the growth factor Wnt-1 in mammary tumorigenesis is demonstrated using transgenic mice which express this gene in the mammary epithelium. These mice develop mammary duct hyperplasias which progress to a malignant phenotype (Kwan *et al.*, 1992). The low penetrance of this phenotype suggests that some additional events are required for malignancy. The introduction of another growth factor, int-2 into the mammary epithelium induces mammary hyperplasias but full malignancy develops infrequently and after a long latency (Muller *et al.*, 1990; Kwan *et al.*, 1992). The stochastic development of tumorigenesis argues that expression of either transgene alone is not sufficient to produce a malignancy. Bigenic mice generated from interbreeding of these two transgenic strains provided the first direct evidence that co-operation between two factors could enhance the effects of either growth factor.

While many studies have identified genes capable of inducing a transformed phenotype in cell lines, this is not always the case *in vivo*. While *myc, ras* and *src* oncogenes are powerful transforming agents *in vitro* (see Weinberg, 1985 for review), examination of the mammary glands of transgenic mice has revealed that their expression alone is not sufficient to induce widespread transformation (Sinn *et al.*, 1987; Stewart *et al.*, 1984).

1.6 Role of receptor tyrosine kinases

Cellular activity is controlled by external signals that either promote or discourage intracellular events. The transmission of an external signal within a cell is termed signal transduction and is usually initiated by the interaction of a ligand (hormones, adhesion

molecules, neurotransmitters) with a receptor on the cell surface. The signal is transmitted to the innerside of the cellular membrane where cytoplasmic domains of the receptors interact with other cellular molecules which can, in turn, stimulate multiple intracellular pathways, each eliciting different effects.

Receptor tyrosine kinases act as relays in the elaborate network that determines the phenotype of a cell. Augmented activity of these receptors can occur because of increased expression or through mutation within the gene product that alters its biochemical properties (Cantley, 1991). As of yet, there is no example of an altered function, such as a change in substrate. There is plenty of evidence, however, for the deregulation of expression of receptor tyrosine kinases or alterations which confer constitutive activity upon the receptor (Bishop, 1991).

1.7 Neu/ErbB-2/Her-2

Neu was initially isolated (Padhy *et al.*, 1982) from chemically induced rat neuroglioblastomas. Sequence analysis revealed that Neu and its human homologue ErbB-2, were structurally related to the epidermal growth factor receptor (EGFR) (Bargmann *et al.*, 1986 a). Sequence comparison of Neu from neurogliobastomas with the wildtype cellular gene revealed a single nucleotide difference, resulting in a non-conservative amino acid change within the transmembrane domain (Bargmann *et al.*, 1986 b). This point mutation confers a constitutive ligand-independent kinase activity (Bargmann and Weinberg, 1988). This activated form will be referred to as Neu^{nt}.

100% of both female and male transgenic mice expressing neu^{nt} develop multifocal mammary tumors (Muller *et al.*, 1988). Histological evaluation revealed multiple dysplastic nodules arising synchronously in the mammary epithelium of virgin females. Within three months, all female carriers had developed multiple mammary carcinomas involving the entire gland. These tumors resembled human comedo-carcinomas known to express elevated levels of Neu and even developed pulmonary metastases, suggesting an excellent model for human carcinogenesis (Cardiff and Muller, 1993). This neu^{nt} data provides evidence for oncogenic ability of the gene and suggests that ErbB-2 was the primary mechanism of tumor formation. However, the one-step progression does not reflect epidemiological data of human breast cancer and isolation of a comparable mutation in human breast tumors has not yet been possible.

To test the effects of overexpression, transgenic mice expressing the *neu* proto-oncogene under transcriptional control of MMTV were generated (Guy *et al.*, 1992b). The female transgenic mice from several independent lines developed focal mammary adenocarcinomas juxtaposed to hyperplastic epithelium after a lengthy latency period. These tumors also resembled human comedo-carcinomas with elevated levels of c-ErbB (Morrison, 1994). Neu protein from tumor lysates displayed elevated levels of kinase activity compared with lysates of adjacent hyperplastic tissue which could not be explained by different levels of protein (Guy *et al.*, 1992b). This data argues that an increased activity of Neu was responsible for the tumors. Because *neu* can be activated by somatic mutations, RNA from breast tumors and adjacent tissue was examined for mutations using RT/PCR and RNase protection assays (Siegel, *et al.*, 1994). The result

identified multiple in-frame deletions within tumors which were not within the adjacent epithelium. Different deletions were detectable even within a single transgenic mouse but the deletions were restricted to separate tumors. The deletions ranged from 5 to 12 amino acids in the extracellular domain proximal to the transmembrane domain (Siegel *et al.*, 1994). The elevated kinase activities supports the idea that the majority of MMTV/*neu* tumors arise as a result of somatic mutations of the transgene. These mutations result in the promotion of dimerization of the Neu through the formation of disulfide bonds, resulting in its constitutive activation (Siegel and Muller, 1996).

Overexpression of c-ErbB2 has been detected in a large proportion of human breast and ovarian carcinomas (Slamon *et al.*, 1998; Hynes and Stern, 1994) and overexpression can be correlated to a poor clinical prognosis (Hynes and Stern, 1994; Mansour *et al.*, 1994). The incidence and level of neu amplification in human breast cancer remains consistent in matched primary and metastatic lesions from the same patient (Lacroix *et al.*, 1989) suggesting amplification is an early event in initiating mammary carcinomas.

Protein –protein interactions are involved at all stages of signal transduction. Growth factor receptors contain distinct binding sites that are able to recruit multiple signaling molecules. $p185^{neu}$ is proposed to transduce signals via at least four pathways associated with the binding of SH2 (src homology domain)-containing proteins to autophosphorylation sites: (i) PLC-γ, (ii)GAP, (iii)PI3'K and (iv)Shc.

1.8 Role of Polyomavirus middle T antigen in tumorigenesis

Another tyrosine kinase implicated in the genesis of murine mammary carcinogenesis is the Polyomavirus (PyV). PyV was initially isolated as a cell-free extract prepared from a leukemic mouse strain that was capable of inducing parotid tumors in AKR or CH3 mouse strains (reviewed by Gross, 1970). Infection of newborn mice with PyV resulted in the generation of a range of mesenchymal tumor types, of which mammary adenocarcinomas represented a significant proportion (reviewed by Dawe *et al.*, 1987). Also, infection of adult female athymic nu/nu mice resulted in mammary tumors with a high frequency while the induction of other tumor types was rare (Haslam *et al.*, 1992). Together, this data suggests that PyV induced transformation of the mammary gland may involve a regulatory pathway similar to those involved in human breast cancers.

The transforming potential of PyV is localized to the early region of the genome (Chowdhury *et al.*, 1980; Hassell *et al.*, 1980). Three different proteins are encoded in the early region and are derived by alternative splicing of a single primary transcript (Treismann *et al.*, 1981). Large tumor antigen is a 100 kD nuclear protein required for viral replication and has been exploited by researchers as a means to immortalize primary cell cultures (Rassoulzadegan, 1983) and alone is weakly oncogenic (Linzer *et al.*, 1979). Small tumor antigen is a 22 kD protein, which has been shown to promote cell growth (Noda *et al.*, 1986). The middle tumor antigen (MT) is a 56 kD membrane phosphoprotein with transforming activity (Ito and Spurr, 1980; Treismann *et al.*, 1981). A functional MT antigen is required for PyV induced tumor formation (Israel *et al.*,

1979). MT antigen is a phosphoprotein of 421 amino acids that resides in the cellular membrane (Smith *et al.*, 1979). MT antigen is anchored into the plasma membrane by 22 hydrophobic amino acids at its carboxyl terminus. Membrane localization is necessary for MT transformation ability as mutants defective in membrane localisation are not able to transform cells (Markland *et al.*, 1986). In PyV infected cells, MT antigen exists as a 56 kD or 58 kD protein, depending upon its state of phosphorylation (Schauffhausen *et al.*, 1981).

While MT possesses no intrinsic biochemical function (Kaplan *et al.*, 1988), it is able to transform a wide variety of cell types by utilizing cellular host proteins and their signaling pathways. The association of Src or Src family members with PyV MT is critical for viral transformation both *in vitro* and *in vivo*. Genetic analysis demonstrates that MT mutants which are incapable of associating with Src, phosphatidyl inositol 3' kinase or Shc are transformation incompetent (Markland and Smith, 1987; Druker *et al.*, 1992; Campbell *et al.*, 1994; Dilworth *et al.*, 1994) suggesting that MT requires the activation of several cellular molecules to mediate transformation.

To directly test the oncogenic potential of MT, transgenic mice carrying a MMTV/PyV MT fusion gene were established. All transgenics which expressed MT in the mammary epithelium developed synchronous, multifocal, mammary tumors with a short latency concurrent with transgene detection (Guy *et al.*, 1992a). Because the tumors in these animals demonstrated a high tendency to metastasize to the lung, this model recapitulated all stages of human breast cancer.

1.9 Overview of Shc

The mammalian gene *shc* encodes three overlapping proteins of 46, 52 and 66 kD ($p46^{Shc}$, $p52^{Shc}$ and $p66^{Shc}$) which contain a C-terminal SH2 domain and an N-terminal collagen-homology region rich in proline and glycine residues (CH1) (Pelicci *et al.*, 1992). p46 and p52 are translated from the same transcript using two different initiation codons while p66 is encoded by an alternatively spliced transcript and contains a unique N-terminal region which is also rich in glycines and prolines (CH2) (Migliaccio *et al.*, 1997). While p46 and p52 are found in all cell types, $p66^{shc}$ expression varies among cell types (Pelicci *et al.* 1992). Nothing is presently known about the mechanism that regulates the expression of the three isoforms *in vivo*.

Increasing evidence indicates that Shc proteins are involved in the intracellular signal transduction from tyrosine kinases to Ras and become phosphorylated on tyrosine upon activation of all RTKs tested thus far: epidermal growth factor receptor (EGFR) (Pelicci *et al.*, 1992); platelet derived growth factor (PDGF) (Yokote *et al.*, 1994); hepatocyte growth factor receptor (HGFR) (Pelicci *et al.*, 1995); erbB-2 receptor (Segatto *et al.*, 1993); insulin receptor (Giorgetti *et al.*, 1994); fibroblast growth factor receptor (Vainikka *et al.*, 1994) and nerve growth factor receptor (Borrello *et al.*, 1994; Stephens *et al.*, 1994). Shc proteins are also involved in signaling non-receptor tyrosine kinases and they are constitutively phosphorylated in cells that express activated Lck, Src, Fps or Sea (McGlade *et al.*, 1994; Dilworth *et al.*, 1994). In addition, Shc proteins are rapidly phosphorylated on tyrosine after ligand stimulation of receptors that have no intrinsic

tyrosine kinase activity but are thought to signal by recruiting and activating cytoplasmic tyrosine kinases (Lanfrancone *et al.*, 1995).

Upon phosphorylation, Shc proteins are able to form stable interactions with other cellular tyrosine-phosphorylated molecules which are facilitated by the Shc SH2 domain (Pelicci *et al.*, 1992; Segatto *et al.*, 1993; Stephens *et al.*, 1994; Yokote *et al.*, 1994; Lanfrancone *et al.*, 1995). Phosphorylated Shc proteins also associate with the Grb2 adaptor protein (Clarke *et al.*, 1992) through direct binding of the Grb2 SH2 domain to the major Shc tyrosine phosphorylation site (Rozakis-Adcock *et al.*, 1992; Salcini *et al.*, 1994). Grb2 is constitutively complexed with SOS, an ubiquitously expressed guanine nucleotide exchange factor for Ras (Simon *et al.*, 1991). Recruitment of the Grb2/SOS complex to Shc proteins relocalizes SOS to the cell membrane, an event considered sufficient for the activation of Ras (Aronheim *et al.*, 1994). This suggests that Shc is involved in the regulation of the Ras pathway.

Involvement of Shc in the Ras pathway is also supported by the fact that overexpression of Shc in neuronal cells induces terminal differentiation. This effect is prevented by coexpression of dominant-negative Ras mutants (Rozakis-Adcock *et al.*, 1992). In addition, overexpression of Shc in NIH3T3 cells induces a transformed phenotype (Pelicci *et al.*, 1992). Shc proteins are constitutively phosphorylated in tumors where known tyrosine kinases are activated, such as EGFR, Met, and ErbB-2 (Pelicci *et al.*, 1995). No constitutive phosphorylation of Shc has been found in primary cell lines or normal tissues (Pelicci *et al.*, 1995). While $p66^{shc}$ does not lack the ability to become phosphorylated after growth factor stimulation, recent data suggests that expression of

p66 is unable to increase MAP kinase activation or transform fibroblasts. In fact, $p66^{shc}$ appears to exert an antagonistic effect on EGF-stimulated fos promoter activity (Migliaccio *et al.*, 1997).

Shc appears to interact with tyrosine-phosphorylated proteins bearing the sequence Asn-Pro-X-Tyr(P). This interaction is unusual in that SH2 domains usually select binding sites based on amino acids carboxyl terminal to the phosphotyrosine (Songyang *et al.*, 1992). A second domain in the amino terminus, the phosphotyrosine binding domain (PTB), distinct from the SH2 domain interacts with tyrosine-phosphorylated growth factor receptors (Blaike *et al.*, 1994). In the case of EGFR, physical interaction of Shc with the receptor is not actually a necessary prerequisite to mediate the phosphorylation of Shc (Egan *et al.*, 1993).

The addition of phosphorylated Shc to unactivated T-cell lysates is sufficient to enhance the interaction of Grb2:SOS (Pelicci *et al.*, 1995), providing evidence that Shc plays a role in regulating the Grb2:SOS interaction. Shc links tyrosine kinases to Ras signaling by recruiting the Grb2-mSOS complex to the plasma membrane in a phosphorylation dependent manner (Pawson, 1995). In most cases, phosphorylated Shc forms a complex with Grb2 at phosphorylated tyrosine 317, the high affinity Grb2 binding site implicated in the activation of Ras (Salcini *et al.*, 1994). The overexpression of Y_{317} mutants not only fails to enhance mitogenicity and motogenicity in response to hepatocyte growth factor (HGF), but causes a slight inhibition (Pelicci *et al.*, 1995).

Recent studies indicate that a subset of β_1 and $\alpha_v\beta_3$ integrins are linked to Ras signaling and immediate early gene expression by Shc. Ligation of these integrins linked

to Shc enabled primary endothelial cells to progress through G1 in response to mitogens. Also, ligation of the laminin receptor $\alpha_6\beta_4$ expressed in basal keratinocytes causes tyrosine phosphorylation of Shc resulting in the recruitment of Grb2, activation of Ras and stimulation of the MAP kinases Erk and Jnk (Mainiero *et al.*, 1997). Ligation of other integrins not associated with Shc, under the same conditions, results in exit from the cell cycle (Mainiero *et al.*, 1997; Wary *et al.*, 1996). These findings suggest that the association of specific integrins with Shc regulates cell survival and cell cycle progression in response to the extracellular matrix.

It also appears that Shc plays a role in the anti-apoptotic function of interleukin-3 (IL-3) (Gotoh *et al.*, 1996). Conditional overexpression of a Shc dominant negative mutant was found to induce apoptosis of IL-3 dependent Ba/F3 cells along with a reduction of c-*myc* gene expression. Apoptosis was rescued by the introduction of exogenous c-*myc*. Tryptic phosphopeptide mapping identified two novel phosphorylation sites of Shc, tyrosines 239 and 240 which were phosphorylated upon stimulation with IL-3 in Ba/F3 cells and in EGF receptor expressing NIH 3T3 cells. Ba/F3 cells expressing the Shc Y317F mutant, which is unable to couple Grb2 and stimulate the Ras pathway, were resistant to apoptosis. However, cells expressing Shc Y239/240F, which is able to stimulate the Ras pathway, were sensitive to apoptosis (Gotoh *et al.*, 1996). This Shc to Myc pathway appears to be distinct from the Ras/MAPK pathway, however, the two pathways may be activated in parallel.

1.10 PyV MT recruits Shc

Middle T antigen (MT) is the principle transforming protein of polyoma virus. Signaling through Ras is necessary for MT transformation. Microinjection of anti-Ras antibodies or a dominant negative Ras mutant is able to prevent growth in MT transformed cells (Jelinek and Hassell, 1992). It appears that binding of the adaptor molecule Shc is important in the transduction of the signal from MT through Ras.

In addition to a major tyrosine phosphorylation site, a minor tyrosine phosphorylation site (Y_{250}) within the MT protein is recently shown to act as a high affinity binding site for the SH2/phosphotyrosine binding domain (PTB) containing protein, Shc (Campbell *et al.*, 1995; Dilworth *et al.*, 1994). Shc becomes phosphorylated on tyrosine following growth factor receptor activation. Tyrosine residue 317 of Shc, contained within the PTB domain, creates a consensus binding site for the SH2 domain of Grb2 (Rozakis-Adcock *et al.*, 1992). Grb2, which is constitutively complexed to the GTP exchange protein SOS, activates Ras. Activated Ras mediates the activation of Raf and proteins involved in the mitogen activated protein kinase (MAPK) pathway (reviewed in Seger *et al.*, 1995). The activation of the MAPK cascades appears to be central to many biological processes, including tumorigenesis.

Previous experiments demonstrate that the Shc/MT interaction is dependent upon the phosphorylation of tyrosine 250 of MT. Mutation of this site to a phenylalanine residue results in a transformation defective MT (Campbell *et al.*, 1994). Transformation can be rescued by reinsertion of the wildtype sequence NPXY further downstream in the MT sequence.

1.11 Experimental rationale

Malignant tumors usually arise from a sequence of events. The common view is that each step in sequence creates an additional phenotype. The steps are broadly defined and each may be a result of a new abnormality within the cell. Because the contribution of Shc to a transformed phenotype is not yet clear, I have attempted to dissect steps and provide evidence to link the steps. An abundance of data has been gathered which shows how lesions in several genes might combine to produce a malignant phenotype (Bishop, 1991). The development of molecular genetic techniques has led to the identification of essential proteins that mediate biological processes and even elucidated signaling pathways responsible for disease.

It is becoming increasingly clear from experimental and clinical observations that activation of tyrosine kinases plays an integral role in the induction of mammary tumors. However, the exact role of certain downstream adaptor molecules, such as Shc, has not yet been determined *in vivo*. While it is understood that Shc is able to complex with multiple tyrosine kinases implicated in tumorigenesis and is constitutively phosphorylated in many human tumor samples (Pelicci *et al.*, 1995), how this molecule controls the activation of mitogenic signaling pathways is not clear. Support for its involvement in tumorigenesis derives from the observation that overexpression of Shc proteins transforms NIH 3T3 fibroblasts (Pelicci *et al.*, 1992).

To determine the role of Shc in mammary tumorigenesis, I have generated transgenic mice which overexpress p52 Shc under the transcriptional control of MMTV LTR. To go a step further and to directly test the role of Shc in the context of a

constitutively activated receptor tyrosine kinase, MMTV/Shc mice were crossed with MMTV/NDL1-2, a constitutively activated form of Neu receptor tyrosine kinase. The phenotype generated from these bigenic mice will enhance our understanding of the molecular basis for the potent transforming ability of constitutively activated Neu.

While it is clear that the interaction of PyV MT antigen with cellular proteins such as Shc is important in tumorigenesis, the relative contribution of this molecule is not completely defined. PyV MT antigen has been demonstrated as the principle transforming agent of Polyomavirus and has been shown to transform the mouse mammary gland (Guy *et al.*, 1992). Using a PyV MT mutant with an impaired ability to bind Shc, it has been demonstrated that MT was still tumorigenic within the mouse mammary gland (Webster *et al.*, submitted to Mol. Cell. Biol.). However, 7% of the tumors that arose had reacquired its capacity to bind Shc through the occurrence of somatic mutations in the transgene. Reversion events occurred at an even higher frequency in lung metastases, suggesting selective pressure to restore Shc binding. The requirement for Shc may reflect its ability to recruit other signaling pathways. I have generated transgenic mice which overexpress MT Y250F and Shc in the mammary gland to enhance our understanding of the role of Shc in mammary tumorigeneis and provide important insight into the molecular basis of metastatic progression.

Chapter 2

Materials and Methods

2.1 DNA constructions.

pMMTVmShc(p52) and pMMTVmShc(p52)Y313F plasmids were obtained from Venus Lai and Tony Pawson (Mount Sinai, Toronto). The mouse mammary tumour virus long terminal repeat (MMTV LTR) promoter extends from the first Bam HI site to the Hind III site. The 5' end of the 1.4 kb mShc cDNA sequence begins at the Hind III site and extends to the EcoRI site. An SV40 polyadenylation signal extends from the 3' end of the Shc cDNA to the fourth Bam HI site.

2.2 Preparation of DNA for microinjection

pMMTVmShc (p52) and pMMTVmShcY313F (p52) plasmid DNAs were amplified by chloramphenicol treatment (Clewell et al., 1972) in *E. coli* DH5-A. Supercoiled molecules were isolated by lysozyme-SDS lysis followed by a cesium chloride (CsCl) density gradient (Clewell and Helsinki, 1972). The resulting 6 kb MMTV/Shc and MMTV/ShcY313F fragments to be microinjected were obtained by cleavage of pMMTV/Shc and pMMTV/ShcY313F with 4U (each) of Sal I and Spe I per ug DNA for 1 hour. The DNA was then electrophoresed through a 1% agarose gel.

Bands containing the injection fragments were excised and the DNA was electroeluted in TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) buffer. The eluates were extracted once with butanol, 4 times with phenol-chloroform (1:1) and twice with chloroform alone. The purified DNA was ethanol precipitated, dried and resuspended in water. DNA quality and concentration was confirmed by electrophoresis of an aliquot onto an agarose gel and run against a standard. The injection stock was diluted to a concentration of 5 ug/ml in sterile water and stored at –20 ^{o}C.

2.3 Isolation of embryos for microinjection

FVB/N female mice (Taconic Farms, Germantown, Pa) were superovulated with two gonadotropins: pregnant mare's serum (PMS; Organon Inc., 5 IU in PBS, injected intraperitoneally 72 hours before embryos were harvested) and human chorionic gonadotropin (Sigma; 5 IU in PBS, injected intraperitoneally 24 hours before the embryos were harvested). The superovulated FVB/N mice were mated with FVB/N male mice 24 hours before harvest of the embryos. FVB mice were chosen for the generation of these transgenic mice because of their low rate of spontaneous tumor formation. One-cell mouse embryos and cumulus were released from the uterine tubules with 45^{o} angle forceps and placed in M2 culture medium (Quinn et al., 1982; 94.66 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2.2H_2O$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4.7H_2O$, 4.15 mM $NaHCO_3$, 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 an approximate

concentration of 300 ug/ml. After removing the cumulus, the embryos were washed in M2 culture medium to remove the hyaluronidase and were then placed on a depression slide and overlaid with paraffin oil. The embryos 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 pronuclei of the zygotes.

2.4 Embryo transfers

Following microinjection, viable embryos (determined by gross observation) were washed once in M2 medium and transferred to the oviducts of pseudopregnant Swiss-Webster mice (Taconic Farms; Germantown, Pa). To obtain pseudopregnant Swiss-Webster mice, females were mated to vasectomised Swiss-Webster (Taconic Farms; Germantown, Pa) males 24 hours prior to embryo transfer. The surgery was performed under a stereoscope using Avertin (Aldrich) as anesthetic (injected intraperitoneally at a concentration of 0.15-0.17 ml/g body weight). Buprenorphine (Temgesic) was given at a dose of 0.01-0.05 mg/kg body weight for relief of pain.

2.5 Identification of transgenic animals

To identify transgenic progeny, genomic DNA was extracted from 1.5 cm tail clippings of weaned pups as described previously by Muller *et al.* (1988). The nucleic acid pellet was resuspended in 100 ul of distilled water to an approximate concentration of 1 ug/ml. 15 ul of the DNA suspension was digested with 30 units of BamHI for 1.5 h.

Following gel electrophoresis and Southern blot transfer (Southern, 1975) onto Gene-Screen filters (Dupont), the blots were hybridized to transgene radiolabeled with [α^{32}P]dCTP (Dupont) by random priming (Feinberg *et al.*, 1983). p206SPA was used to generate an antisense probe to identify the SV40 polyadenylation sequence of both MMTV/Shc and MMTV/Shc Y313F founder animals.

For F1 generations and beyond, all transgenic mice (MMTV/Shc, MMTV/Shc Y313F, MMTV/MT Y250F, MMTV/Shc/MT Y250F bigenics, MMTV/NDL1-2, MMTV/Shc/NDL1-2 bigenics) were identified using polymerase chain reaction (PCR) to detect the presence of transgene.

The PCR reactions contained 1 ug DNA, 10 mM Tris pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at 200 uM, reverse and forward oligonucleotide primers at 0.01 ug/ul and 0.03 U/ul Taq polymerase (Promega), double distilled water in a total reaction volume of 25 ul. The reactions were overlaid with mineral oil to prevent evaporation during thermal cycling

For *shc* transgenics, forward and reverse primers #6926 (GGA CAT GAA CAAGCT GAG TGC) and #6927 (GAC GCA AAA GAG ATA GAT TGC) were used in the reaction and were cycled 35 times (94°, 1 min; 92°, 30 sec; 60°, 30 sec; 72°, 1 min; 72°, 5 min). Mice carrying the *neu* transgene were identified using primers #8736 (TTC CGG AAC CCA CAT CAG GCC) and #8737 (GT TTC CTG CAG CCT ACG C).The reaction was cycled 30 times (94°, 1 min; 92°, 30 sec; 58°, 30 sec; 72°, 1 min; 72°, 5 min). The middle T transgene was detected with primers #6515 (GGA AGC AAG TAC TTC ACA AGG G) and #6516 (GGA AAG TCA CTA GGA GCA GGG) and cycled 35 times

(94°, 1 min; 92°, 30 sec; 55°, 30 sec; 72°, 1 min; 72°, 5 min). All oligonucleotide primers were synthesized at the MOBIX Central Facility on an Applied Biosystems oligonucleotide synthesizer.

2.6 RNA analysis

Tissue RNA was isolated by the procedure described by Chirgwin *et al.* (1979) using the CsCl sedimentation gradient modification. The tissues were dissected from the animals and flash frozen in liquid nitrogen and stored at –80 °C or immediately homogenized with a polytron (Texmar Company, Ohio, USA) in 3 ml guanidine isothiocyanate (GIT, BRL) solution containing 4 M GIT, 25 mM sodium citrate, and 0.1M β-mercaptoethanol. The tissue homogenate was layered onto 4 ml of 5.7 M CsCl containing 25 mM sodium acetate (pH 5.2). RNA was pelleted by ultracentrifugation at 32 000 rpm at 20 °C using an SW41Ti rotor (Beckman) for approximately 18 h. GIT and CsCl phases were aspirated from the tube and the RNA pellet was resuspended in 100 ul of resuspension buffer (5 mM EDTA, 0.5% sarkosyl, 5% β-mercaptoethanol) and 400 ul sterile water and precipitated with 2 volumes ice cold ethanol. Yield was determined by UV absorption at 260 nm, 1 OD_{260}=40 ug/ml RNA.

Ribonucleotide protection probes were made with either pSP64/65 or pSL301 (Invitrogen) vectors prepared by restriction digest with blunt end or 5'-OH overhangs. Linearized riboprobe was analysed by agarose gel electrophoresis to ensure digest was complete. The linearized DNA was extracted from the agarose using NaI/glass milk purification (Geneclean, Biocan) and resuspended in 50 ul sterile water. 1 ug of each

template was used as template for an *in vitro* transcription assay. Briefly, 30-60 U of corresponding RNA polymerase (pSP64/65:SP6 polymerase [BRL]; pSL301:T7 polymerase [BRL]) was added to 25 ul of transcription cocktail (200 mM Tris pH 7.5, 30 mM MgCl2, 20 mM Spermidine, 15 mM DTT, 40 U RNase inhibitor RNasin [Boehringer Mannheim], 100 uCi [β-32P] UTP [10 mCi/ml, 3000 mCi/mmol], 1 mM each of rCTP, rGTP,rATP and 0.1 mM rUTP. The reaction was incubated for 45 min at 37°C. The reaction was quick chilled on ice and another 30-60 U of RNA polymerase was added. The reaction was allowed to proceed for another 30 min. at 37 °C. Transcription was terminated by the destruction of the DNA template by the addition of 20 U of RNase-free DNaseI, 1 ul of 0.5 mM MgCl2 and 20 ul sterile water and incubated at 37 °C for 10 min. After bringing the volume to 100 ul with sterile water, an equal volume of phenol:chloroform (1:1) was added. The aqueous phase was extracted and precipitated with the addition of 300 mM sodium acetate (pH 5.2), 20 ug RNase-free tRNA (MRE 600, Boehringer Mannheim) and 2-4 volumes ethanol. After centrifugation and aspiration of the ethanol, the probe was resuspended in 100 ul sterile water. Percent radiolabel incorporation was assessed by spotting in duplicate 1 ul of each riboprobe onto Whatman-DE81 ion exchange filters. One filter was washed one time each with 5 ml of 0.5 M sodium phosphate buffer, water and ethanol to remove unincorporated nucleotide. Both filters were then placed in scintillation cocktails (Beckman) and radioactivity was measured by a scintillation counter (Beckman).

RNase protection assays were performed as described by Melton *et al.* (1984) using 20 ug total cellular RNA per tissue incubated with 1 ul prepared riboprobe in

hybridization buffer (80% formamide, 40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) pH 6.4, 1 mM EDTA pH 8.0, and 400 mM NaCl) at 85 °C for 5 min. after which the temperature was reduced to 50 °C. RNA was allowed to anneal overnight at 50 °C. Hybridization mixtures were then quick-chilled and spun at 4 °C. RNA digestion conditions was as follows: 300 ul RNase digestion buffer (300 mM NaCl, 10 mM Tris pH 7.4, 5 mM EDTA pH 8.0, 2 ug/ml RNase T1, and 40 ug/ml RNase A) was added and incubated for 20 min. at room temperature. Digestion was terminated by the addition of 20-30 ug RNase-free yeast tRNA and 500 ul phenol:chloroform (1:1) followed by ethanol precipitation. Pelleted RNA was dried completely and resuspended in 10 ul of formamide sample loading buffer (80% formamide, 10 mM EDTA pH 8.0, 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue). The samples were boiled for 5 min at 95 °C and resolved on a 6% urea polyacrylamide gel (40% acrylamide:2%N,N'-methylene-bis-acrylamide, 7M urea, 0.001% ammonium persulfate and 0.0005% N,N,N',N'-tetramethylethylenediamine (TEMED) which was electrophoresed at 60-80 amps in .6X TBE running buffer (0.1 M Tris, 0.08 M boric acid, 0.002 M EDTA pH 8.0). The gel was dried and exposed at –80 °C against Kodak XAR-5 film in the presence of intensifying screens.

2.7 Antibodies

The antibodies used include a Shc-specific rabbit monoclonal (Transduction, Cat# S14620) for immunoprecipitating and a rabbit polyclonal Shc antibody (Transduction, Cat# S14630) for immunoblotting. Antisera specific for phosphorylated tyrosine

residues-PY20 (Transduction, Cat# P11120) and a Grb2 monoclonal antibody (UBI, Cat#06-304) were used for immunoblotting.

2.8 Protein extract preparation

Tissue samples for protein analysis were flash frozen in liquid nitrogen and ground to a fine powder with a chilled mortar and pestle. The cells were lysed with Triple Detergent Lysis Buffer (50 mM Tris.Cl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate with 1ul/ml 1 M sodium orthovanadate, 10 ug/ml aprotinin and 10 ug/ml leupeptin) for 30 minutes on ice with agitation. The lysates were cleared twice using centrifugation at 13 000x g for 5 minutes each. The supernatants were collected and protein concentration was determined using the Bradford assay kit (Biorad).

2.9 Immunoblotting

A total of 100 ug total protein lysate for each sample was analysed unless otherwise specified. Protein lysates were boiled for 10 minutes at 95°C with an equal volume of 2X protein sample loading buffer (62.5 mM Tris pH6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue). Proteins were resolved on SDS-polyacrylamide gels and electrophoretically transferred (Biorad) onto polyvinylidine difluoride (PVDF) membranes (Immobilon-P, Millipore). The membranes were blocked overnight in 3% powdered skim milk in TBS (20 mM Tris pH7.5, 150 mM NaCl, 5 mM KCl) or 3% BSA (Fraction IV, Sigma) in TBS for anti-

phosphotyrosine immunoblots. Antibodies were added to the blocking agent and incubated for 2 hours at room temperature. All monoclonal antibodies were prepared at a dilution of 1:1000 while polyclonal antibodies were diluted to 1:250. Membranes were washed three times for 10 minutes each in TBS with 0.01% Tween-20 (TBST) and once in TBS for five minutes before incubation for 1 hour at room temperature with the appropriate secondary antibody conjugated to horseradish peroxidase (HRP, Biocan Scientific) at 1:2500-1:5000 dilution. The membranes were washed three times in TBST and once in TBS before proteins were detected using the enhanced chemiluminescence detection system (ECL, Amersham) and Kodak XAR-5 film.

2.10 Immunoprecipitations

Immunoprecipitations were performed by preincubating an antigen-specific antibody (1-2 ug monoclonal; 5-10 ug polyclonal) with 30-40 ul of Protein G Sepharose fast flow (Pharmacia) in 800 ul 1XPBS (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4) for 2-12 hours at 4°C on a rotating platform. Antibody-bound sepharose beads were washed once with one ml 1XPBS and then once with 1 ml lysis buffer. 500 ug of total protein lysate was added to a total volume of 700 ul and incubated with the antibody-bound sepharose beads for 1-5 hours at 4°C on a rotating platform. The beads were then washed 4 times with lysis buffer and once with PBS and resuspended in 70 ul of 2X protein sample loading buffer. All samples were denatured at 95°C for 10 minutes before resolution on SDS-polyacrylamide gels, transferred onto PVDF membranes, and blotted with the appropriate antibodies.

2.11 Histological evaluation

The development of mammary tumours was detected by twice-weekly palpations of the 10 mouse mammary fat pads. Upper left mammary fat pads (fat pad #3L) were removed from sacrificed animals and fixed in 4% paraformaldehyde overnight and dehydrated in 70% ethanol. Specimens were blocked in paraffin, sectioned to 5 um and stained with hematoxylin and eosin (performed by the Department of Pathology, McMaster University). Diagnosis was confirmed by a pathologist, Dr. Robert Cardiff of the University of California, Davis.

Wholemount preparations were prepared using the upper right mammary fat pad (#3R) as described by Vonderhaar *et al.* (1979). The resected tissue was spread on a glass slide and allowed to air dry overnight. The glands were then defatted overnight in acetone. To enhance the defatting process, the glands were squeezed between 2 slides in the morning and replaced in fresh acetone for another hour before the glands were stained overnight in Harris modified hematoxylin. The tissues were destained in several changes of destain solution (1% HCl in 75% ethanol) until the epithelial component of the mammary gland was seen in sharp contrast to the light background of the fat pad. The stain was fixed with a 30 second wash in 0.002% ammonium hydroxide. The slides were transferred to 75% ethanol for 1 hour and then to 100% ethanol for several more hours to dehydrate the tissue. The glands were cleared overnight in xylenes and then mounted with Permount (Fisher Scientific Co.) and a coverslip was placed over the tissue.

2.12 Thin sectioning

Tissue for thin section histological evaluation was fixed overnight in 4% paraformaldehyde in PBS. After rinsing the specimens in 70% ethanol, they were stored in 70% ethanol. Thin sectioning, hematoxylin and eosin staining, and mounting were performed by Pathology Research Services, McMaster Hospital.

Chapter 3

Results

3.1 Introduction

$p46^{Shc}$ and $p52^{Shc}$ are ubiquitous cytoplasmic proteins which act as downstream targets and general effectors of protein tyrosine kinases. Shc proteins become tyrosine phosphorylated upon activation of receptor tyrosine kinases (Pelicci *et al.*, 1992; Yokote *et al.*, 1994; Pelicci *et al.*, 1995; Segatto *et al.*, 1993; Giorgetti *et al.*, 1994; Borello *et al.*, 1994) and are implicated in the regulation of the Ras pathway. Overexpression of Shc proteins in neuronal cells induces terminal differentiation, while coexpression of Ras dominant negative mutants prevents this effect (Rozakis-Adcock *et al.*, 1992). Overexpression of Shc in fibroblasts or myeloid cells increases the proliferative response and mitogen-activated (MAP) kinase activation by EGF. When overexpressed in NIH 3T3 cells, Shc is able to induce transformation (Pelicci *et al.*, 1992).

Tyrosine phosphorylated Shc proteins form a complex with the SH2-SH3-containing Grb2 protein, which is implicated in the regulation of Ras. This suggests that Shc is directly involved in the intracellular transmission of signals from tyrosine kinases to Ras. Tyrosine 317 (compares to Y_{313} in mouse) is the major, but not only, site of phosphorylation on Shc, and is a major binding site for Grb2. Mutant Shc proteins with

substitution of Y_{317} by phenylalanine reduce their capacity to bind Grb2 and reduces neoplastic transformation *in vitro* (Salcini *et al.*, 1994). Because of a substitution of a tyrosine residue at position 317 with a phenyalanine residue (317F) in the glycine/proline-rich domain, this mutant version of Shc is limited in its ability to complex Grb2 and activate the Ras pathway (Salcini *et al.*, 1994). To directly test the oncogenic potential of overexpression of $p52^{mouse\ Shc}$ as well as the effect of overexpression of a putative dominant Shc gene in the mammary epithelium, transgenic carrying MMTV/Shc and MMTV/Shc Y313F fusion genes were derived.

As well as evaluating the role of Shc in the development of normal mammary epithelium , we sought to explore the role of Shc in two established mouse mammary tumor models. The *neu* (c-*ErbB-2* or *Her2*) proto-oncogene encodes a receptor tyrosine kinase belonging to the epidermal growth factor receptor family (Bargmann *et al.*, 1986; Coussens *et al.*,1985). Activation of Neu through somatic in-frame deletions in the extracellular region proximal to the transmembrane domain results in an increased transforming ability over that of Neu activated by a point mutation (Siegel *et al.*, 1994). Because Shc has been shown to associate with this receptor (Segatto *et al.*, 1993), we investigated the effect of overexpression of Shc on this tumor model.

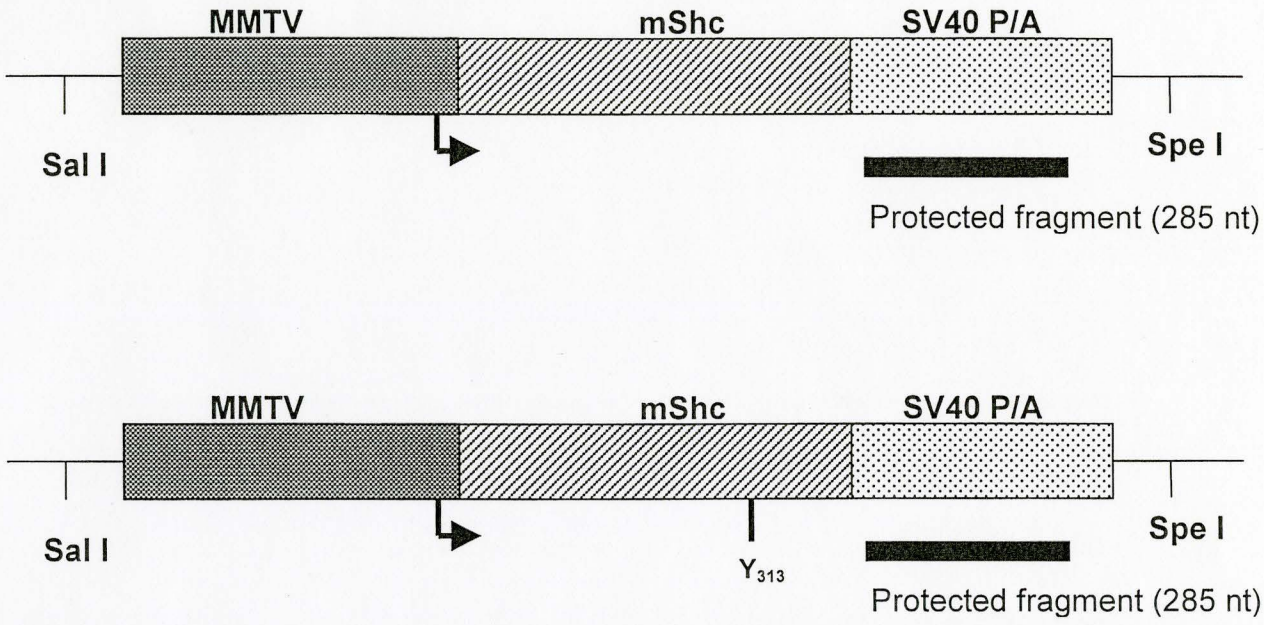
Similar to the activated Neu receptor tyrosine kinase, Polyomavirus middle T antigen (MT) complexes the protein Shc. MT is able to efficiently transform the mouse mammary epithelium, however, functional inactivation of the binding site for Shc (Y250) debilitates its transforming ability. Because a large proportion of MT Y250F lung metastases demonstrate the ability to reacquire the Shc binding site (Webster *et al.*,

Figure 3.1 Transgene constructs used in the generation of MMTV/Shc and MMTV/Shc Y313F transgenic mice and tissue specificity of transgene expression.

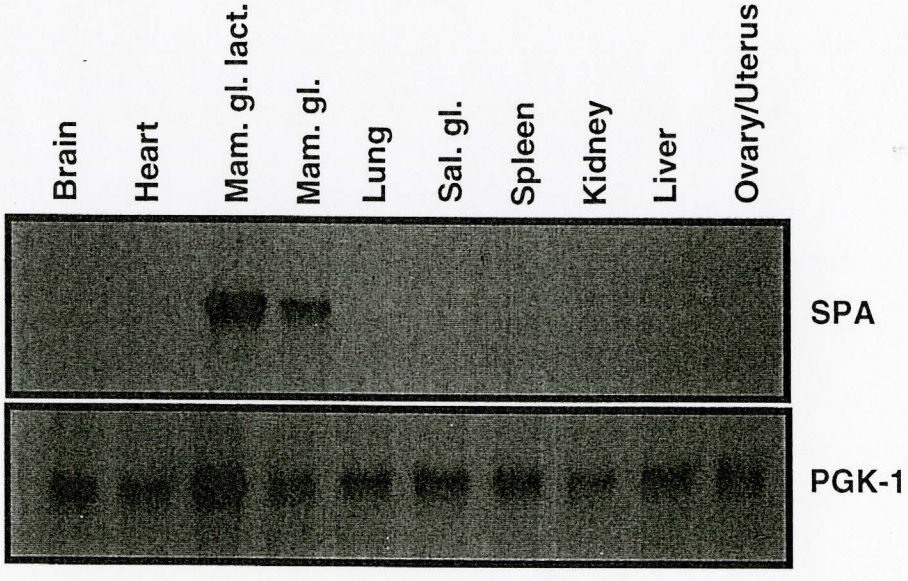
(A) Structure of the pMMTV/Shc and pMMTV/Shc Y313F expression vectors. Vector backbone sequence represented by the single line refers to sequences from Bluescript SK (Promega). The grey area corresponds to the Mouse Mammary Tumor Virus-Long Terminal Repeat. The slashed region indicates the mouse Shc p52 cDNA. The stippled area 3' to the mShc cDNA refers to the SV40-derived polyadenylation/splicing sequences. Intact circular plasmid (obtained from Tony Pawson) was restricted with Sal I and Spe I to produce a linear injection fragment.

(B) RNA transcripts corresponding to the MMTV/Shc transgene were detected in various organs of transgenic strains as assessed by RNase protection. Tissues were derived from virgin and lactating female mice. The antisense probe (SPA) used in this analysis protects a 285 nucleotide fragment corresponding to the SV40-derived polyadenylation/splicing sequences. Also shown is an RNase protection performed on the same RNA samples with an antisense probe directed to phosphoglycerate kinase which protects a 124 nt fragment indicated by PGK and an arrow.

A


MMTV
mShc
SV40 P/A
Sal I
Spe I
Protected fragment (285 nt)
MMTV
mShc
SV40 P/A
Sal I
Spe I
Y_{313}
Protected fragment (285 nt)

B


Brain
Heart
Mam. gl. lact.
Mam. gl.
Lung
Sal. gl.
Spleen
Kidney
Liver
Ovary/Uterus
SPA
PGK-1

submitted to Mol. Cell. Biol.) we sought to determine whether overexpression of Shc was capable of rescuing the Polyomavirus middle T (MT) mutant deficient in its ability to bind Shc.

3.2 Generation and tissue site expression of MMTV/Shc and MMTV/Shc Y313F transgenic mouse strains

To provide high levels of transcription of $p52^{Shc}$ and $p52^{Shc\ Y313F}$ in the mouse mammary epithelium, the transgenes were placed under the transcriptional control of the MMTV LTR (Figures 3.1A). The MMTV LTR is expressed throughout all stages of mammary gland development with increased levels during gestation (Muller, 1991), enabling us to examine the effect of transgene expression throughout mammary gland development.

Of nine MMTV/Shc founder animals generated, one male failed to reproduce and one female founder failed to pass the transgene to her progeny in a Mendelian fashion. Eight MMTV/Shc Y313F founders were generated, however, one male and one female both failed to produce any progeny.

To assess the tissue specificity of transgene expression in the remaining seven MMTV/Shc lines (Table 1) and six MMTV/Shc Y313F lines (Table 2), 20 ug of total RNA from various tissues of transgenic mice were subjected to an RNase protection assay with pSPA, a transgene specific probe to the SV40 polyadenylation –splicing signal. This probe protects a 285 nucleotide fragment

Table 1 Transgene expression in MMTV/Shc transgenic lines

Twenty micrograms of total cellular RNA isolated from a variety of tissues from male and female transgenic mice were analysed for expression of transgene by RNase protection analyses. The riboprobe used (SPA; Figure 3.1) is directed toward the MMTV LTR component of the transgene and yields a 285 bp protected fragment. Relative levels of transgene expression are indicated by +/- (low) to +++ (high).

Table 1. Transgene expression in MMTV/Shc transgenic lines

Line	V. M.gl	L. M.gl	Sal. gl	Br.	Lu.	H	Liv	Spl	Sm. Int	Kid	Ov. Ut.	Test.	Sem Ves
Shc1	-	-	-	-	-	-	-	-	-	-	-	-	-
Shc2	-	-	-	-	-	-	-	-	-	-	-	-	-
Shc3	++	+++	+/-	-	-	-	-	-	-	-	-	-	-
Shc4	-	-	-	-	-	-	-	-	-	-	-	-	-
Shc5	-	-	-	-	-	-	-	-	-	-	-	-	-
Shc6	-	-	-	-	-	-	-	-	-	-	-	-	-
Shc7	-	-	-	-	-	-	-	-	-	-	-	-	-
Shc8	-	-	-	-	-	-	-	-	-	-	-	-	-
Shc9	+	++	-	-	-	-	-	-	-	-	-	-	-

V.M.gl=virgin mammary gland; LM.gl=lactating mammary gland; Sal. gl=salivary gland; Br.=brain; Liv=liver; Spl=spleen; Sm. int.=small intestine; Kid.=kidney; Ov/Ut.=ovary and uterus; Test.=testes; Sem. Ves.=seminal vesicles

Table 2 Transgene expression in MMTV/Shc Y313F transgenic lines

Twenty micrograms of total cellular RNA isolated from a variety of tissues from male and female transgenic mice were analysed for expression of transgene by RNase protection analyses. The riboprobe used (SPA; Figure 3.1) is directed toward the MMTV LTR component of the transgene and yields a 285 bp protected fragment. Levels of transgene expression are not detected in any of the tissues of these transgenic lines.

Table 2. Transgene expression in MMTV/ShcY313F transgenic lines

Line	V. M.gl	L. M.gl	Sal. gl	Br.	Lu.	H	Liv	Spl	Sm. Int	Kid	Ov. Ut.	Test.	Sem Ves
313-1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
313-2	-	-	-	-	-	-	-	-	-	-	-	-	-
313-3	-	-	-	-	-	-	-	-	-	-	-	-	-
313-4	-	-	-	-	-	-	-	-	-	-	-	-	-
313-5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
313-6	-	-	-	-	-	-	-	-	-	-	-	-	-
313-7	-	-	-	-	-	-	-	-	-	-	-	-	-
313-8	-	-	-	-	-	-	-	-	-	-	-	-	-

V.M.gl=virgin mammary gland; L.M.gl=lactating mammary gland; Sal. gl.=salivary gland; Br.=brain; Lu.=lung; H.=heart; Liv.=liver; Spl.=spleen; Sm. int.=smallintestine;Kid.=kidney; Ov/Ut=ovaries and uterus; Test=testes; Sem. Ves.=seminal vesicles

corresponding to a transcribed non-coding region (Figure 3.1 [Muller *et al.*, 1988]). Representative results from these assays for MMTV/Shc is shown in Figure 3.1B. Results for MMTV/Shc Y313F are not shown.

RNase protection results revealed that the major site of the MMTV/Shc transgene expression was the mammary gland of virgin and lactating female mice. Lower amounts of transgene mRNA were detected in the salivary glands following a much longer exposure. Consistent with this result, a similar tissue distribution was observed in one other independent MMTV/Shc transgenic line (Table 1). Shc Y313F expression could not be detected in any of the six putative dominant negative transgenic strains. It is possible that this mutation is prenatally lethal. While signaling through Ras would still be possible through direct binding of Grb2 to growth factor receptors or novel alternate Grb2 binding sites on Shc (Gotoh *et al.*, 1996; van der Geer, *et. al.*, 1996), the number of other pathways which could be affected are unknown. To address this issue, generation of MMTV/Shc Y313F mice should be attempted again.

3.3 Mammary epithelial-specific expression of MMTV/Shc transgene induces epithelial hyperplasias and rarely focal mammary tumors

Although expression of the Shc transcript in virgin mammary epithelium of mice did not initially induce mammary tumors, histological evaluation of developing mammary glands indicated that terminal buds were enlarged and the numbers increased in MMTV/Shc lines compared to age matched FVB animals (Figure 3.2). Lactation

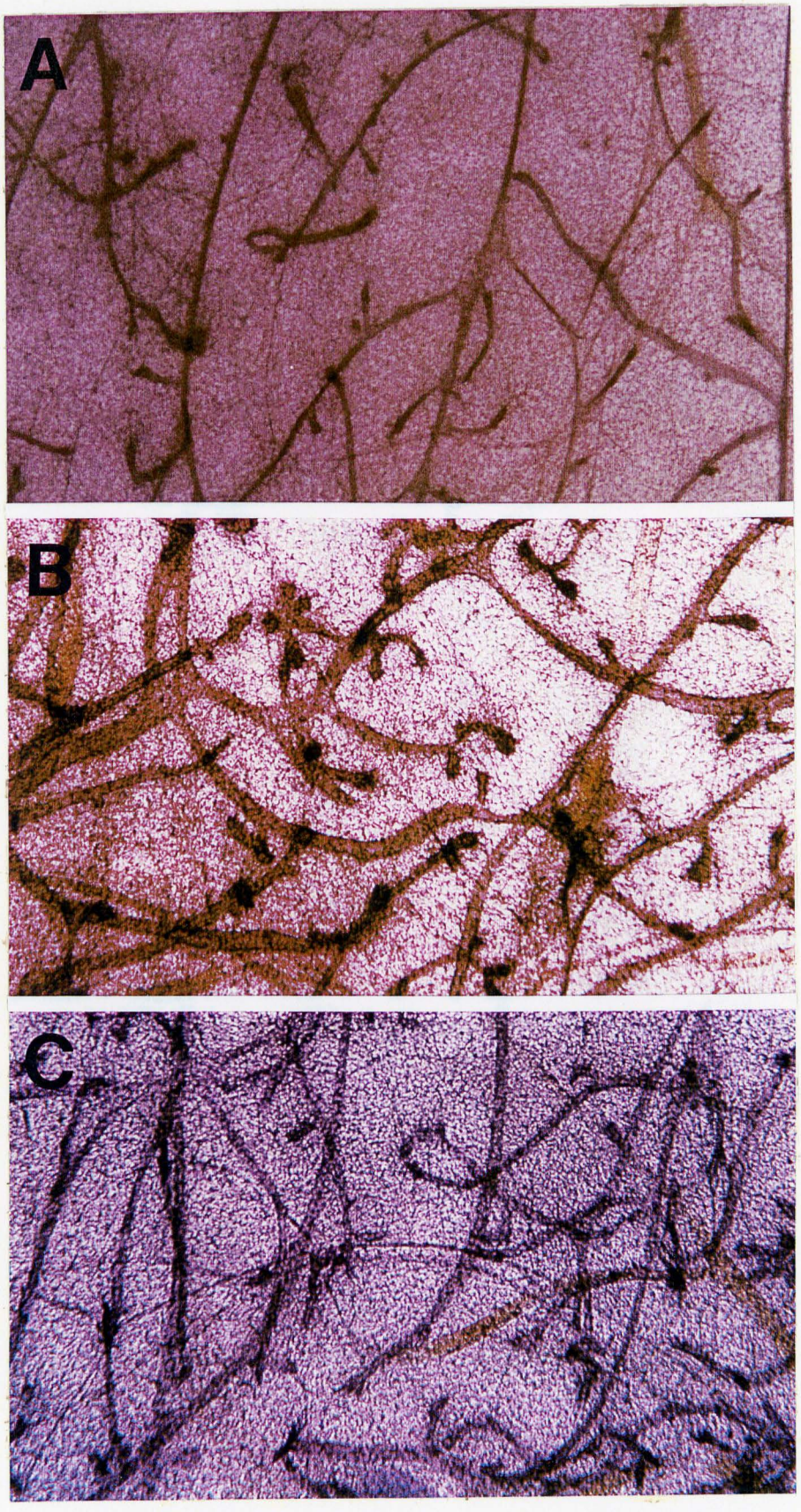
Figure 3.2 Subgross morphology of developing MMTV/Shc mammary glands.

Photomicrographs of hematoxylin and eosin-stained wholemount preparations of eight week old developing female mammary glands. Notice the dilated ducts and terminal buds of increased number and size compared with the control tissue. Magnification, 25X.

(A) FVB

(B) MMTV/Shc3

(C) MMTV/Shc9.


A
B
C

deficiencies were not detected in any of the reproducing females and there was no evidence to suggest that the mammary gland function was impaired or affected in another manner.

Of the virgin mice observed (n=25), none developed tumors during the period of observation (18 months of age). However, epithelial hyperplasias were detected in multiparous females. These hyperplasias became more severe as the mice aged. Histological examination of the mammary fat pads revealed multiple focal epithelial hyperplasias that resembled hyperplastic alveolar nodules (HANs) found in MMTV infected mice (Morris *et al.*, 1990) (Figure 3.3B). The majority of these hyperplasias did not progress to full malignancy, however, after multiple litters, two females (15%) (n=13) from one line did develop multifocal mammary tumors at 296 and 510 days respectively (Figure 3.4). Histological examination of these tumors revealed focal papillary mammary adenocarcinomas with abundant stroma within the papillae. The tumors were surrounded by hyperplastic mammary epithelium.

Histological examination of multiparous mammary glands revealed that mammary glands expressing the Shc transgene failed to regress after the pups were weaned. In an attempt to determine at what point regression was abnormal, histological analysis of mammary glands was performed at day 1 (data not shown), day 10 (data not shown) and day 16 post partum (Figure 3.5). These analyses revealed that mammary gland regression was abnormal following the first litter. After two or more litters had been weaned, Shc transgenic mice displayed extensive lobular-alveolar development with normal acini filled with lipid-laden milk. Mammary ducts appeared dilated (Figure 3.3C

and D). There was distortion of the mammary gland but no evidence of neoplasia in many of the multiparous Shc animals. The residual lobular-alveolar development suggests that overexpression of Shc in the mammary gland prevents normal mammary involution and the effect may be cumulative after multiple litters.

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3.4 Biochemical characterization of MMTV Shc transgenic mammary epithelium

To establish whether expression of transgene transcript in mammary epithelium resulted in a corresponding increase in Shc protein and how these levels related to levels of the protein in the mammary tumors, Western blot analyses with Shc specific antibodies were performed on protein extracts of normal and neoplastic tissue. 100 ug of protein was isolated from mammary tumors, and virgin Shc and virgin FVB mammary epithelium. Representative results for tumor and matched virgin Shc and FVB mammary epithelium are shown in Figure 3.6A. While there was some variation of protein levels in virgin Shc mammary epithelium, the tumors consistently expressed higher levels of Shc protein compared to normal Shc or FVB epithelium suggesting that the effects may be dose dependent.

Because the transforming potential of Shc *in vitro* is related to its state of phosphorylation and because Shc is consistently phosphorylated in some human cancers, we were interested in comparing the levels of phosphorylation in normal and neoplastic tissue. To accomplish this, protein extracts from normal and tumor tissues were immunoprecipitated with monoclonal antibodies specific to mouse Shc and then blotted

Figure 3.3 Histolopathology of multiparous MMTV/Shc transgenic mice.

(A) Hematoxylin-stained wholemount preparation of a multiparous FVB (litter matched) mammary gland. Magnification, 25X.

(B) Hematoxylin-stained whole mount preparation of multiparous MMTV/Shc3 transgenic mammary gland. The distorted gland displays residual lobular alveolar development typical of a regressing pattern. The major ducts are dilated. Most of the side branches end in spiculated tips. Hyperplastic focal lobules are present. Magnification, 25X.

(C) Hematoxylin and eosin-stained thin section of a multiparous FVB (litter matched) mammary gland. Magnification, 100X.

(D) Hematoxylin and eosin-stained thin section of a multiparous MMTV/Shc3 transgenic mammary gland. Extensive lobular-alveolar development is present with normal acini filled with lipid-laden milk. Magnification, 100X.

A
B
C
D

Figure 3.4 Histopathology of MMTV/Shc mammary tumor.

Papillary mammary adenocarcinoma of multiparous MMTV/Shc3 transgenic mouse. The stroma of the papillae is very abundant. The papillae are lined by cells with pleiomorphic, light nuclei and scanty cytoplasm. The tumor contains many areas of necrosis and acutely inflamed debris. Magnification, 100X.

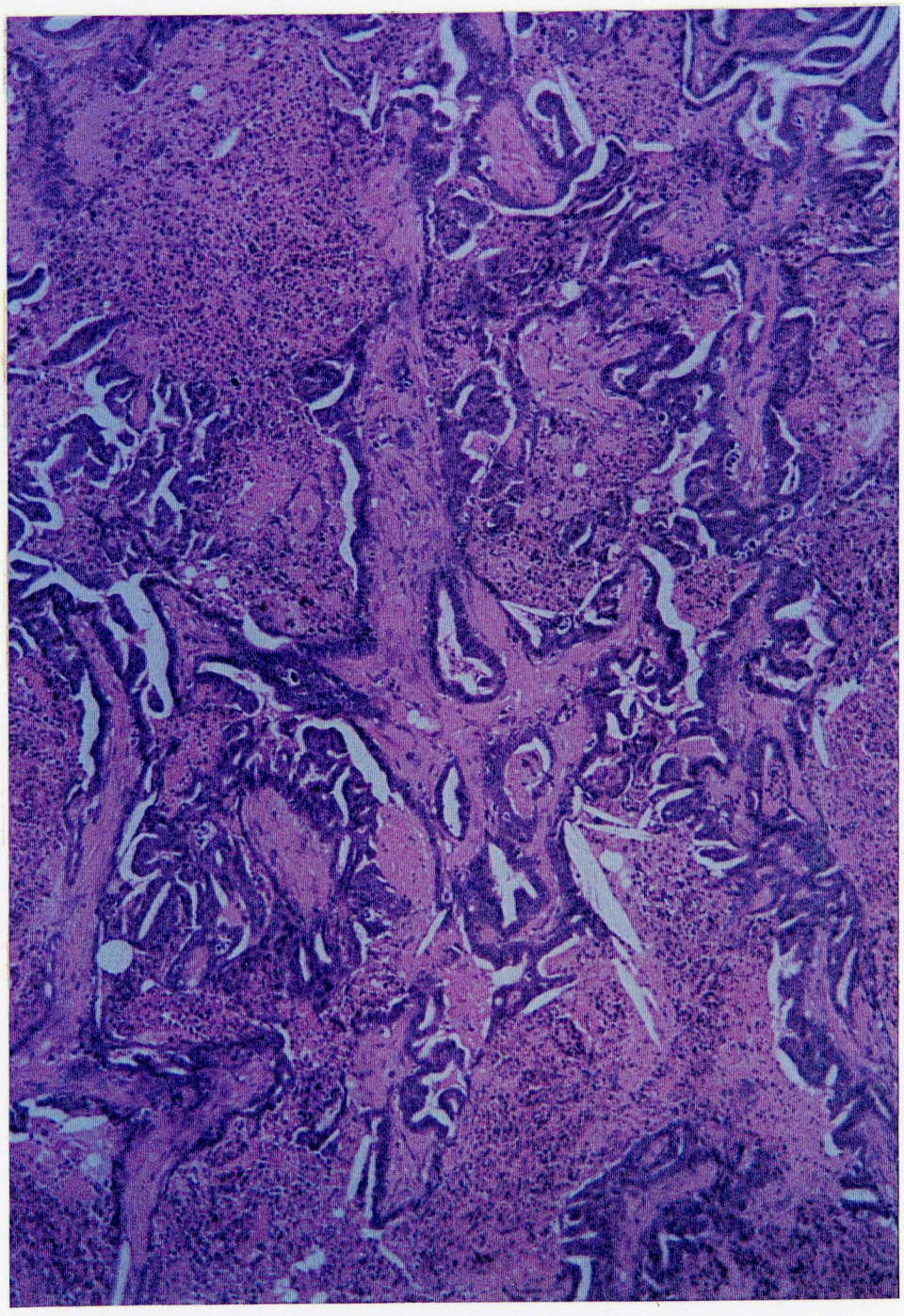
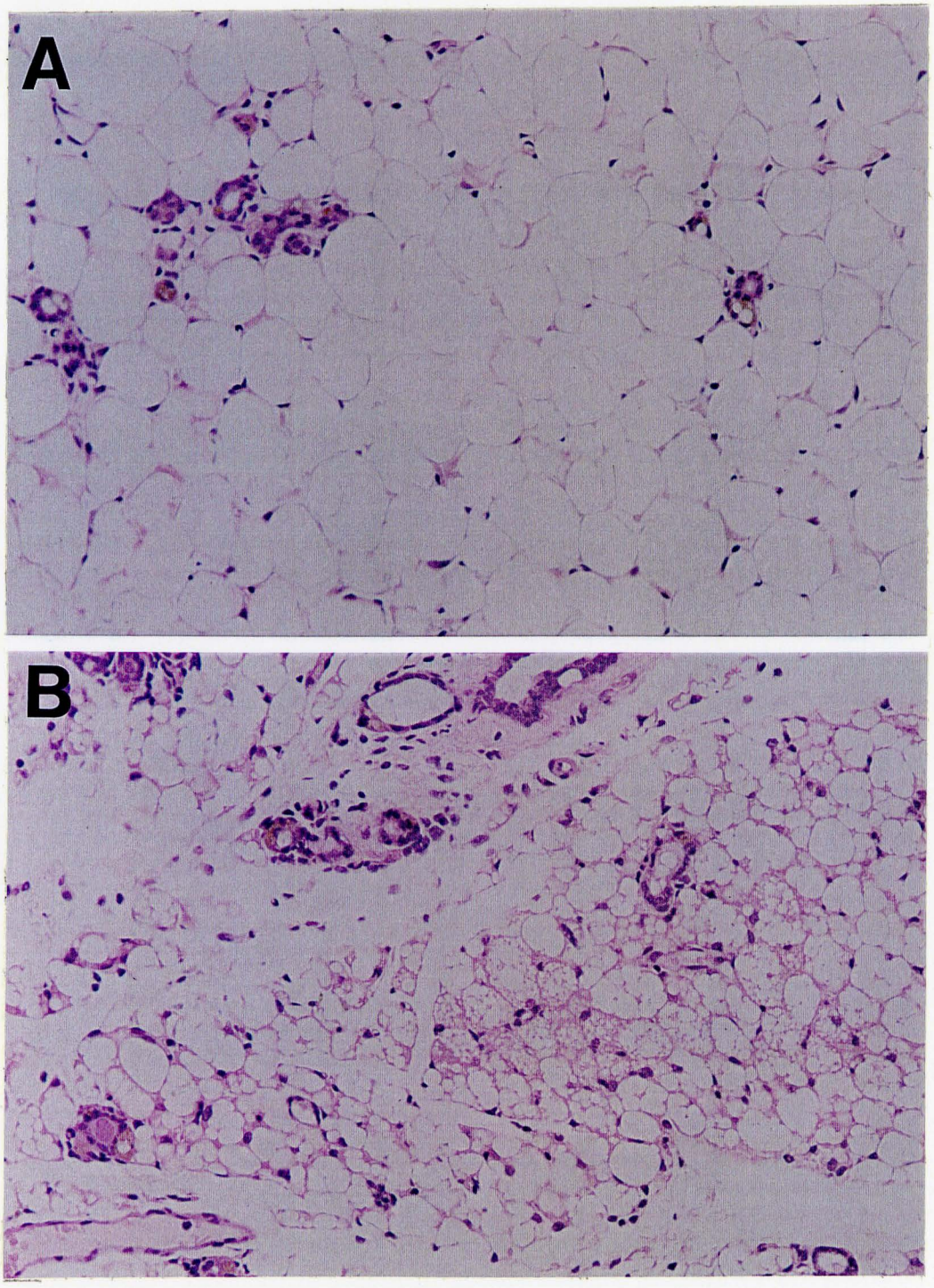


Figure 3.5 Histopathology of mammary gland regression

Hematoxylin and eosin stained thin sections of mammary epithelium 16 days post partum, first litter. Magnification, 200X.

(A) FVB (age matched)

(B) MMTV/Shc


A
B

with an antibody specific to phosphorylated tyrosine (Figure 3.6B). Phosphorylated bands of 46 and 52 kD were detected in both mammary tumor samples. In contrast, little phosphorylation was detected in FVB or virgin Shc mammary glands. Because Shc protein was detected in the epithelium of virgin Shc transgenic animals (Figure 3.6A), these observations suggest that the phenotypes experienced may be related not only to levels of Shc, but also the level of phosphorylation on tyrosine.

3.5 Synchronous expression of MMTV/Shc and MMTV/NDL1-2

Because Shc associates with activated Neu and lies downstream of this receptor tyrosine kinase, we sought to investigate the role of Shc in the context of an established mouse mammary tumor model. Constitutive activation of the Neu receptor tyrosine kinase induces mammary carcinomas in mice when placed under transcriptional control of MMTV LTR and expressed in the mouse mammary epithelium (Muller *et al.*, 1988). Amplification of the Neu/c-ErbB-2 receptor tyrosine kinase has also been implicated as an important event in the genesis of human breast cancer.

To directly test the role of Shc in the context of a constitutively activated receptor tyrosine kinase, mice which overexpress Shc in the mammary epithelium were crossed with mice expressing an activated form of the Neu receptor tyrosine kinase, NDL (neu deletion). The phenotype generated from this cross will enhance our understanding of the molecular basis for the potent transforming ability of NDL and provide insight into the role of the adaptor molecule Shc in signal transduction.

Figure 3.6 Immunoblot analysis of Shc expression in transgene-expressing tissues.

(A) Western analyses of control, normal (Shc 3 virgin) and tumor (Shc 3 BT) tissue from transgenic animals with a Shc-specific monoclonal antibody. Control tissue was isolated from mammary glands of eight week old FVB female. The 46 and 52 kDa proteins are indicated.

(B) Tumor (Shc 3 BT) and non-tumor (Shc 3) tissue lysates were immunoprecipitated with a Shc-specific antibody and blotted with a phosphotyrosine-specific antibody. Phosphorylated 46 and 52 kDa proteins are indicated.

A

FVB
Shc 3
Shc 3 BT-1
Shc 3 BT-2

B

FVB
FVB mp
Shc 3
Shc 3 mp
Shc3 BT
IP:Shc
blot:P-Tyr

To accomplish this, MMTV/Shc/NDL bigenic mice were generated by crossing heterozygous MMTV/Shc female mice with heterozygous MMTV/NDL1-2 males. Bigenic mice were determined with polymerase chain reaction, as described in Chapter 2. Expression of MMTV/NDL1-2 results in the stochastic appearance of tumors in fifty percent (T_{50}) of the animals observed by 259 days (Figure 3.7). Virgin female transgenic mice from the Shc/NDL1-2 bigenic line developed stochastic mammary tumors with an accelerated onset (T_{50}=200 days) compared to NDL controls. It is particularly interesting to note that the Shc/NDL bigenic line resulted in a penetrance of 82% while the NDL control group experienced a penetrance of only 65% during the 18 month period of observation. Despite the difference in tumor kinetics, it is interesting to note that although there is a lot of variability in tumor phenotypes, the tumors of the Shc/NDL mice were not consistently different than those experienced by the control group. The NDL mammary carcinomas most often presented as tumors containing large nodular masses of intermediate cells with uniform, large nuclei (Figure 3.8) while tumors that developed in the Shc/NDL mice were variable and resembled that of either the Neu tumor phenotype or the Ras type tumor. The fact that the Shc/NDL tumors did not resemble the Shc type tumor may be explained by the fact that Shc lies downstream of the Neu receptor and Shc appeared only weakly oncogenic in our previous experiments.

3.6 Synchronous expression of MMTV/Shc and MMTV/MT Y250F

Mammary epithelial expression of the PyV middle T antigen (MT) in transgenic mice results in the induction of multifocal mammary tumors with a penetrance of 100%

Figure 3.7 Mammary tumor onset in MMTV/NDL1-2 and MMTV/Shc/NDL1-2 transgenic strains.

The age at which mammary tumors are first palpable in virgin females of either transgenic strain is indicated. The number of animals analysed in each strain (n) as well as the median (T_{50}) age at which tumors are detectable are also indicated.

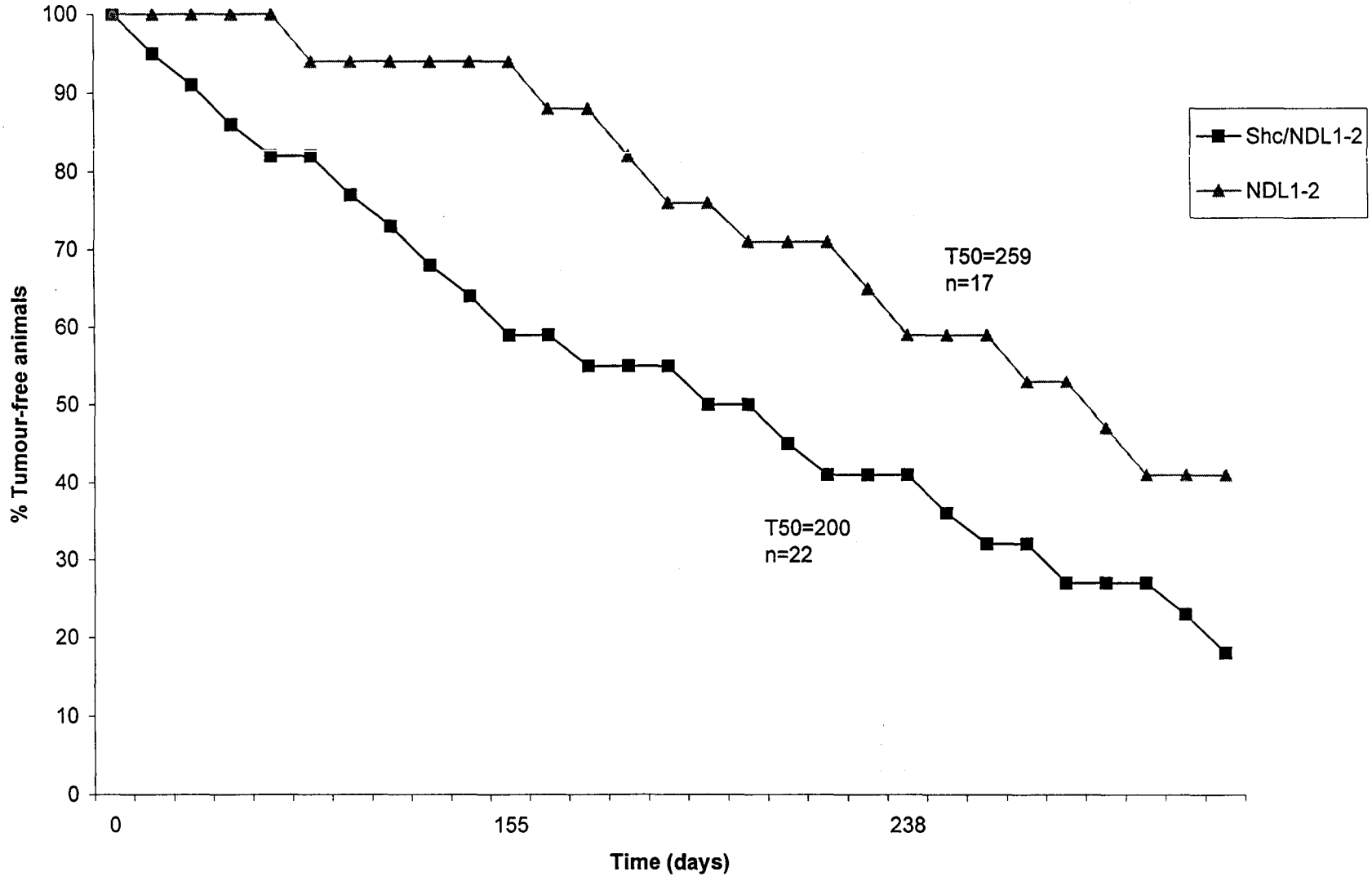
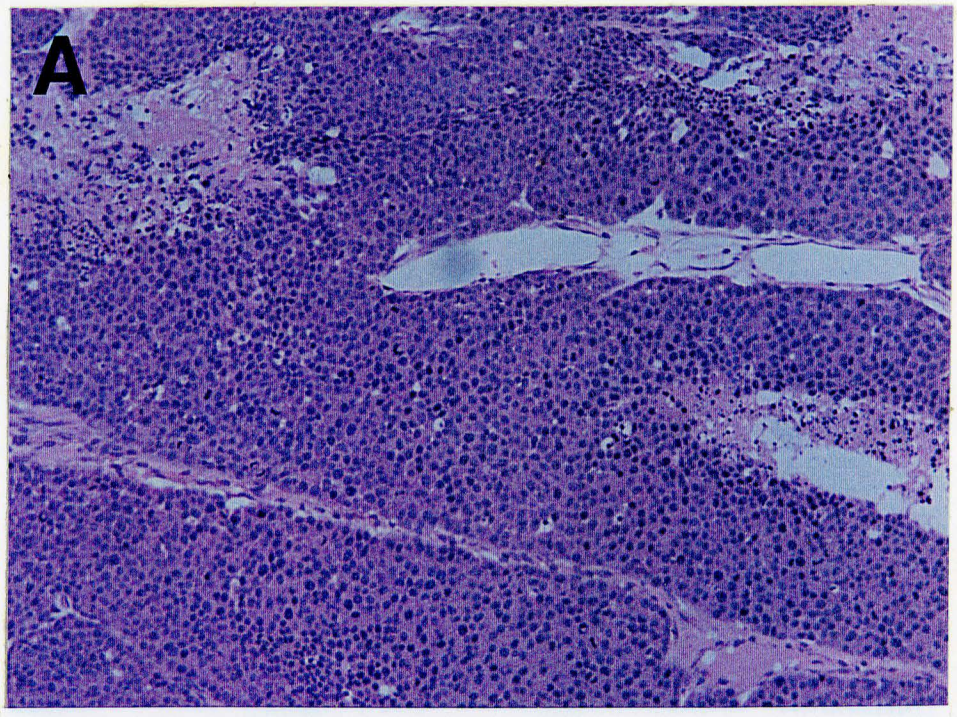

100
90
80
70
60
50
40
30
20
10
0
% Tumour-free animals
0
155
238
Time (days)
Shc/NDL1-2
NDL1-2
T50=259
n=17
T50=200
n=22

Figure 3.8 Histopathology of mammary tumors derived from MMTV/NDL and MMTV/Shc/NDL.

(A) Hematoxylin and eosin stained thin section of a MMTV/NDL mammary tumor from a virgin female. This tumor contains large nodular masses with large nuclei. Magnification, 100X.

(B) Hematoxylin and eosin stained thin section of a mammary tumor from a virgin MMTV/Shc/NDL female. The tumor has large nodular masses of intermediate and small cells with uniform nuclei and a large stromal component. The tumor cytology more resembles the Ras than the Neu type tumor. Magnification, 100X.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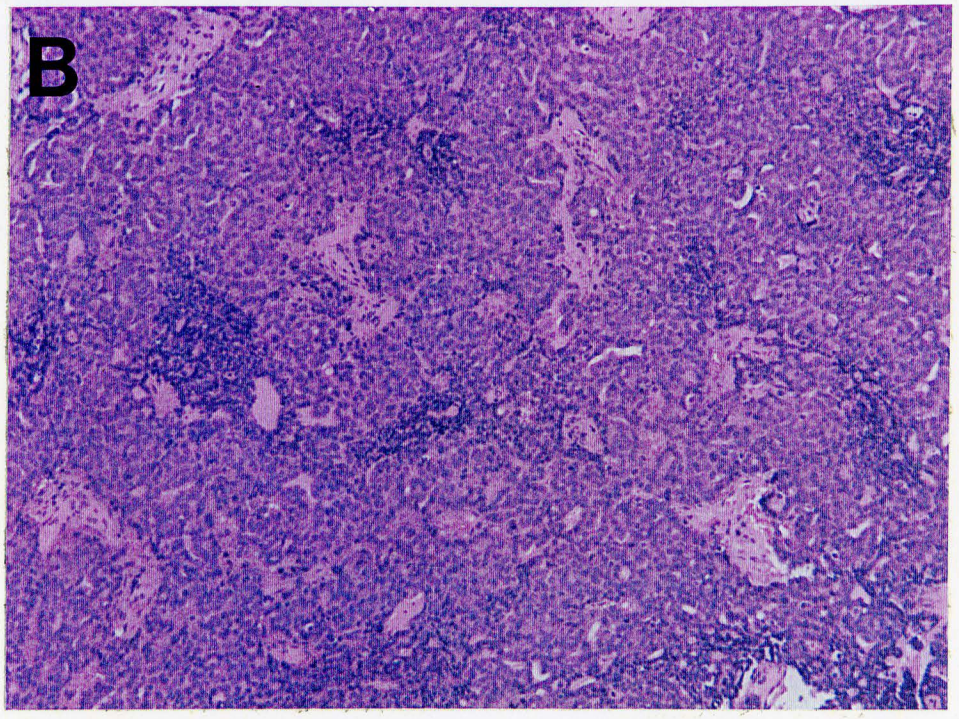

B

by 53 days in 50% (T_{50}=53 days) of the animals observed (Webster, *et al.*, submitted to Mol. Cell. Biol.). These tumor-bearing mice frequently develop pulmonary metastases (Guy *et al.*, 1992a). The potent transforming ability of MT is due to its ability to associate and activate a number of cellular signaling proteins involved in proliferative signal transduction. Tyrosine phosphorylation of MT residue 250, together with residues immediately upstream (247-250), create a high affinity binding site for the PTB domain of Shc (Campbell *et al.*, 1995; Dilworth *et al.*, 1994). Deletion or substitution of this site destabilises the interaction of Shc with MT and severely impairs the ability of MT to transform (Druker *et al.*, 1992). Because phosphorylation on tyrosine 250 is required for the interaction of Shc with PyV MT (Campbell *et al.*, 1994; Dilworth *et al.*, 1994), a tyrosine to phenylalanine substitution at this site ablates Shc binding without affecting MT interaction with other signaling pathways.

Transgenic mice expressing the MT Y250F mutation in the mammary epithelium still develop tumors but with a much later onset. (T_{50}=143 days). Of the tumors that arose in the parental MT Y250F strains, it is interesting to note that 7% of the tumors had reacquired the capacity to bind Shc through the occurrence of somatic mutations in the MT transgene (Webster *et al.* submitted to Mol. Cell. Biol.), suggesting a selective pressure for the binding of Shc.

This mutant model presented us with the opportunity to address the significance of Shc in MT-mediated mammary tumorigenesis and determine whether Shc was able to rescue this mutation by influencing selective pressures to reacquire the Shc binding site. To address this, we have generated bigenic mice which express both MT Y250F and Shc

under the transcriptional control of the MMTV LTR. Bigenic animals were identified using polymerase chain reaction as described in Chapter 2.

The initial phenotype displayed by MT Y250F control and Shc/MT Y250F animals were mammary epithelial hyperplasias. Both strains displayed abnormal and hyperplastic mammary epithelium (Figure 3.9), however, there was a remarkable difference in the extent of hyperplastic development between the two strains. By 10 weeks of age, the Shc/MT Y250F strain had already developed premalignant hyperplastic alveolar nodules (HANs), (Figure 3.9B). Both lines did eventually develop mammary tumors with a penetrance of 100%. Comparison of the tumor kinetics between MT Y250F and Shc/MT Y250F (Figure 3.10) revealed that there is a subtle acceleration in the ability of Shc/MT Y250F to form tumors. Consistent with previous observations, the T_{50} for the MT Y250F control group was 155 days while 50% of the Shc/MT Y250F bigenic animals developed tumors at 125 days. Histological comparison of the tumors displayed subtle differences in the cellular architecture (Figure 3.11). Both tumor types appeared as cystic and hemorrhagic and contained significant neoplastic stromal components. However, there was some variation among the Shc/MT Y250F tumors. In two of the 20 Shc/MT Y250F mammary tumors, the bulk of the tumors were composed of neoplastic spindle cells with a high mitotic rate and attenuated ductal structures which coursed through the stroma. This was an unusual combination not seen in the control animals. This unique spindle cell phenotype was also visible in some lung metastases of these animals.

Figure 3.9 Mammary tumor onset in MMTV/MT Y250F and MMTV/Shc/MT Y250F transgenic strains.

The age at which a mammary tumor is first palpable in virgin females of either strain is indicated. The number of animals analysed in each strain (n) as well as the median (T_{50}) age at which tumors are detectable are also indicated.

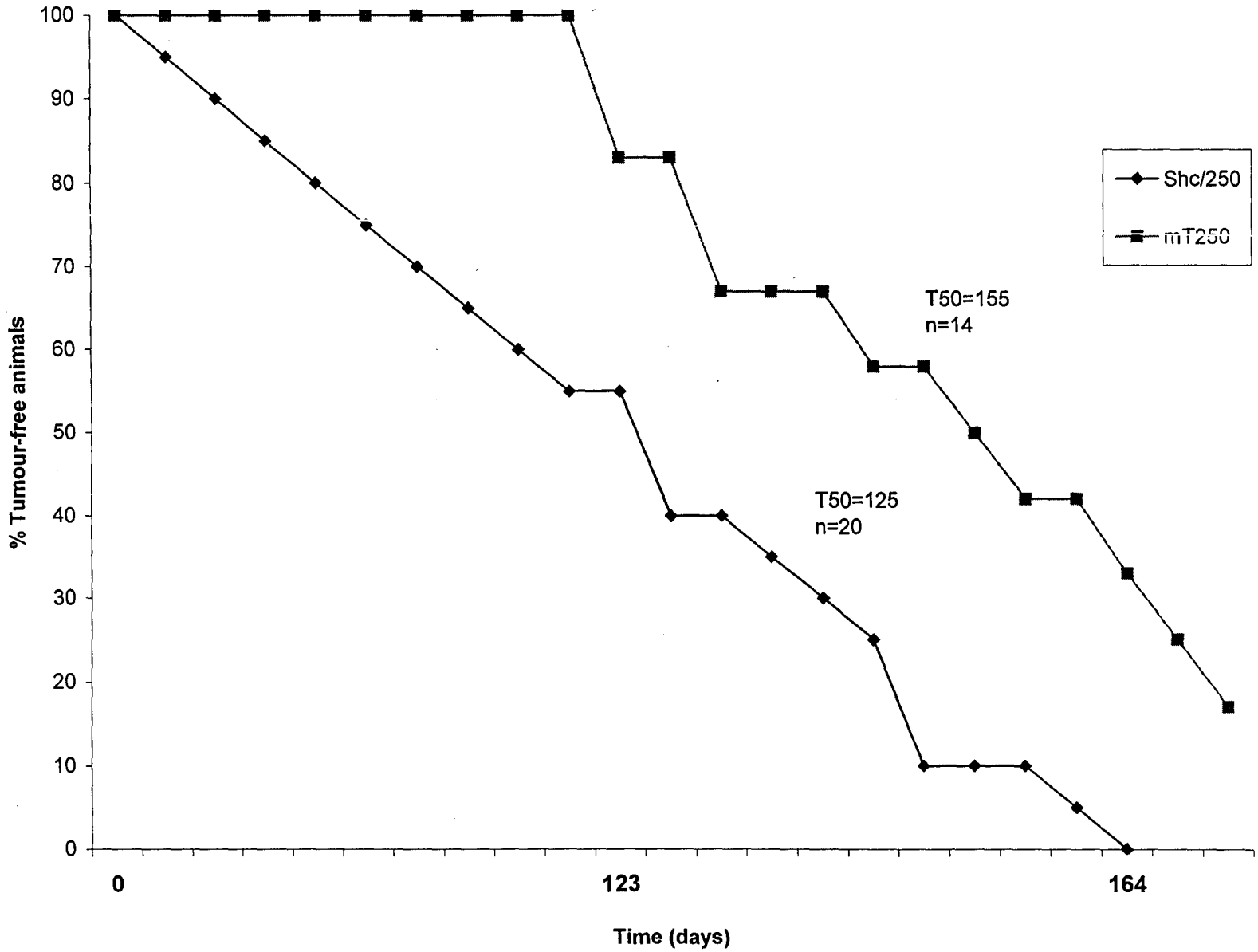

100
90
80
70
60
50
40
30
20
10
0
% Tumour-free animals
0
123
164
Time (days)
Shc/250
mT250
T50=155
n=14
T50=125
n=20

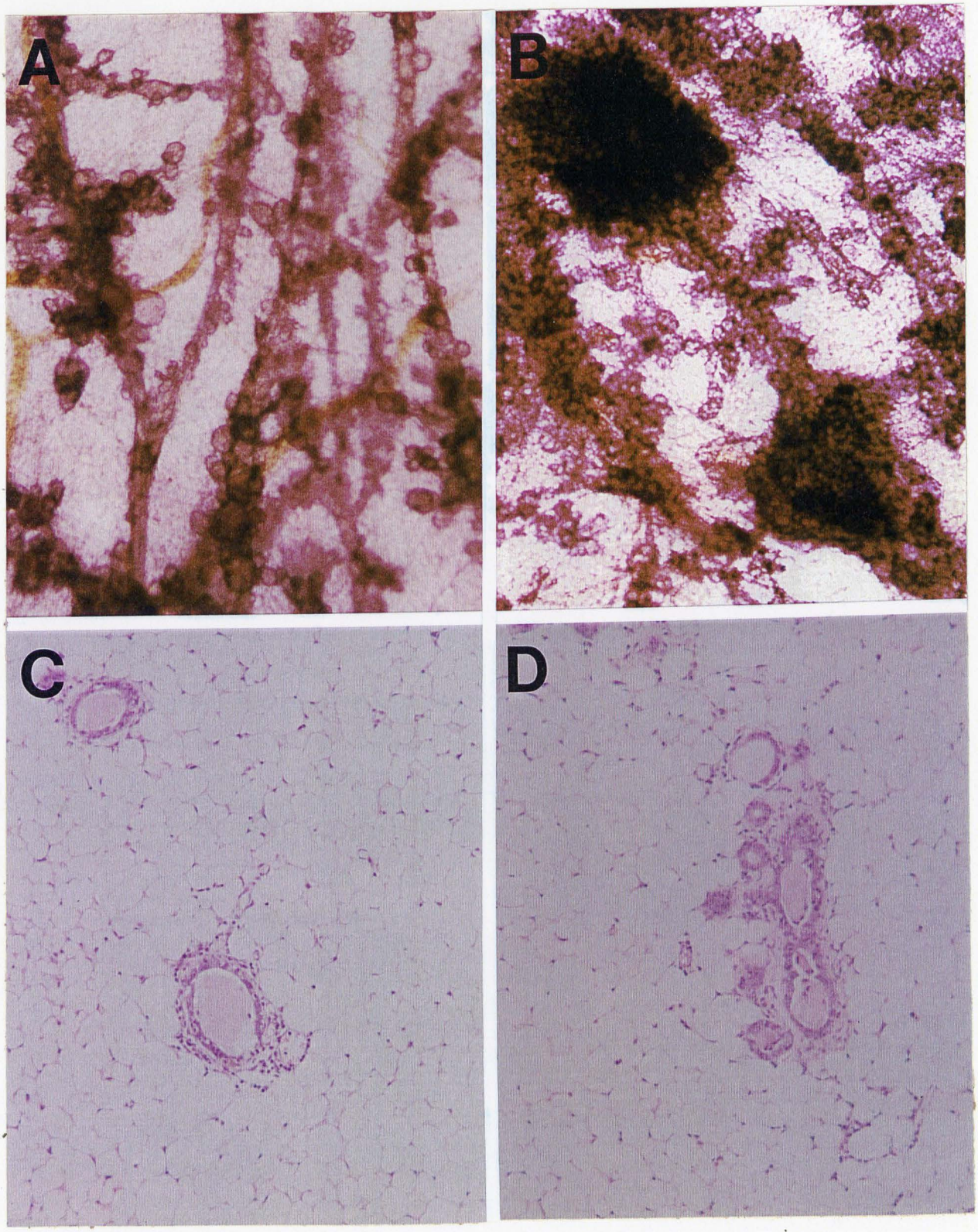
Figure 3.10 Histopathology of developing mammary glands of MMTV/MT Y250F and MMTV/Shc/MT Y250F transgenic strains.

(A) Hematoxylin-stained wholemount preparation of a 10 week old MMTV/MT Y250F female mammary gland. Magnification, 25X.

(B) Hematoxylin-stained wholemount preparation of a 10 week old MMTV/Shc/MT Y250F female mammary gland. Magnification, 25X.

(C) Hematoxylin and eosin-stained thin section of a 10 week old MMTV/MT Y250F female mammary gland. Magnification, 100X.

(D) Hematoxylin and eosin-stained thin section of a 10 week old mammary gland from a female MMTV/Shc/MT Y250F mouse. Magnification, 100X.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To establish whether similar rates of pulmonary metastases occurred in these two transgenic lines, lungs of many tumor-bearing animals were examined at necropsy. In the MT Y250F control group, 20% of the animals examined (n=7) had developed multiple foci of metastatic mammary adenocarcinomas. In contrast, the extent of metastatic involvement in the Shc/MT Y250F bigenic animals was remarkable with respect to penetrance. In the bigenic line, 86% of tumor-bearing animals examined (n=10) had developed metastatic disease at the time of necropsy and were detected as early as 68 days following palpation of the primary mammary tumor. In some cases, entire regions of the lung had been obliterated by pulmonary metastases.

While meaningful comparison of these metastatic rates is complicated by different tumor kinetic profiles (Figure 3.10), these observations suggest that overexpression of Shc in the MT Y250F background promoted a unique metastatic phenotype. Typically, lung metastases observed in the Shc/MT Y250F bigenic line resulted in multiple small foci of neoplastic glands within vessels, parenchyma and pleura. It was interesting to note that some of these poorly organised glands produced lipid droplets (Figure 3.11D) while typically transformed cells do not produce. Taken together, these results suggest that overexpression of Shc in this model affects tumor progression and metastasis by activation of PyV MT-associated signaling pathways.

Figure 3.11 Histopathology of mammary tumors and lung metastases derived from MMTV/MT Y250F and MMTV/Shc/MT Y250F transgenic strains.

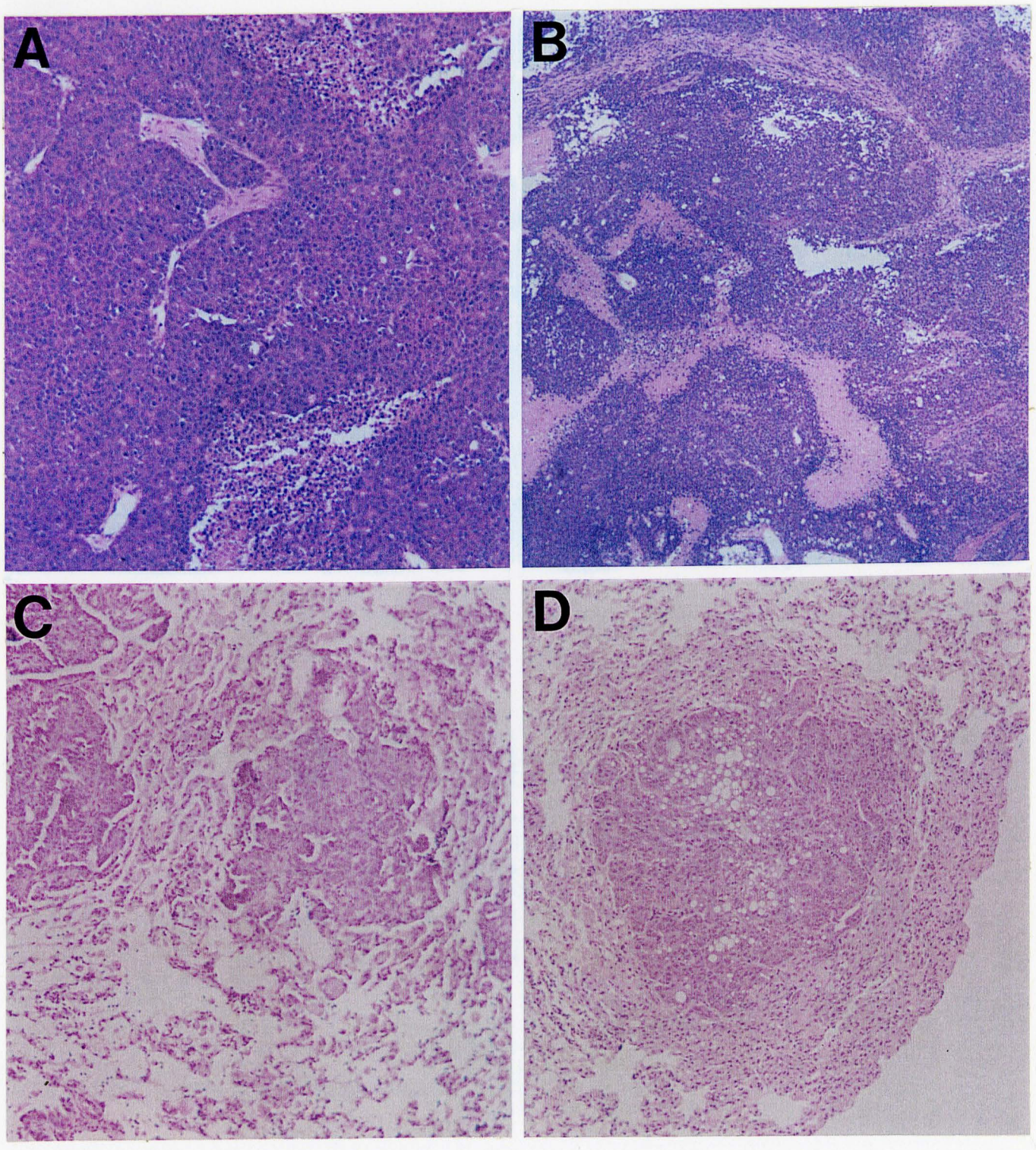
A panel of slide-mounted, hematoxylin and eosin stained sections showing typical tumor morphologies.

(A) Mammary tumor. MMTV/MT Y250F transgenic virgin female. Note the fibrosis associated with this mixed mammary adenocarcinoma. Magnification, 100X.

(B) Mammary tumor. MMTV/Shc/MT Y250F bigenic virgin female. Note the small, pale nuclei. Magnification, 100X.

(C) Lung metastases. MMTV/MT Y250F transgenic virgin female. Magnification, 100X.

(D) Lung metastasis. MMTV/Shc/MT Y250F bigenic virgin female. Note the lipid droplets within the tumor. Magnification, 100X.


A
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C
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Chapter 4

Summary and Discussion

4.1 Overexpression of Shc disrupts normal mammary gland development

Overexpression of Shc in fibroblasts is capable of inducing a transformed phenotype (Pelicci *et al.*, 1992). Shc proteins are constitutively phosphorylated and complexed with activated tyrosine kinases in human tumour cells with known tyrosine kinase gene alterations (Pelicci *et al.*, 1995). Transgenic mice which overexpress Shc provide an excellent model to allow identification of the molecules important in signaling pathways involved in mammary tumourigenesis. Given these facts, I sought to exploit the transgenic mouse model system to elucidate the importance of Shc and its involvement in the signal transduction pathways responsible for mammary gland development and tumourigenesis.

Our observations provide *in vivo* evidence that the SH2-containing protein Shc, a cytoplasmic adaptor whose precise biological role is yet undetermined, has a significant role in mammary development and tumourigenesis. The consequence of overexpression of the Shc transgene in the mammary epithelium provides important insight into the process of malignant progression.

Consistent with previous studies of MMTV-driven transgenes, analyses of the tissue-specific pattern of p52 mouse Shc mRNAs revealed that the primary site of expression was the mammary gland with secondary sites of expression in the salivary glands (Figure 3.1B and Table 1). However, analysis of six p52 Shc Y313F strains revealed that the mutant Shc cDNA was not expressed in any of the tissues examined (Table 2). This was an unexpected outcome. Typically, more than one expressing line would be detected from eight founder animals. One possible explanation for this is that overexpression of this mutant Shc is prenatally lethal. While the expression of this putative dominant negative Shc is not lethal to cultured cells (Pelicci *et al.*, 1995), it is possible that ablation of this Grb2 binding site reduces signaling through Ras to the point where it becomes lethal *in vivo*. Also, receptor tyrosine kinases are able to stimulate Ras through at least two routes. One is through the direct binding of the Grb2-mSOS complex to the autophosphorylated receptor via the SH2 domain of Grb2 (Rozakis-Adcock *et al.*, 1993). The other mechanism is through the phosphorylated Shc-Grb2-mSOS complex (Gotoh, *et al.*, 1995; Rozakis-Adcock *et al.*, 1992). Because these two signaling pathways seem to redundantly activate Ras (Gotoh, *et al.*, 1995), it seems highly unlikely that the expression of Shc Y313F mutant would be prenatally lethal.

More recently, mutagenesis studies indicate that Y239/240 makes an important contribution to the association of Shc with Grb2 (van der Geer *et al.*, 1996; Gotoh *et al.*, 1997; Harmer and DeFranco, 1997) suggesting that the Shc Y313F mutant does not prohibit all Grb2 binding and mitogenic signaling. While it is unusual that from eight founders an expressing line was not generated, to effectively address the issue, another

attempt at generating transgenic mice expressing MMTV/Shc313F should be performed. While *in vitro* studies indicate that Y239/240 of Shc seems to contribute very little to Ras/MAPK activation (Gotoh *et al.*, 1997), the generation of transgenic mice expressing mutations at this Grb2 binding site would help examine the role of Shc in other signaling systems.

In both p52 Shc transgenic lines, our initial observations of virgin female mice indicated abnormal mammary gland development. Wholemount analyses of the mammary epithelium revealed enlarged ducts and terminal buds by eight weeks of age in contrast to age-matched wildtype animals (Figure 3.2). Although we could detect expression of *Shc* encoded protein and RNA in the mammary glands, none of these developmental abnormalities interfered with the ability to lactate. Also, unlike overexpression of Shc in mouse fibroblasts, which develop a transformed phenotype and the development of tumours in nude mice (Pelicci *et al.*, 1992), the multiple enlarged end buds observed in the Shc virgin gland did not progress to hyperplasias.. This observation suggests that overexpression of Shc promotes a weak proliferative signal.

4.2 Expression of Shc in multiparous mice

Although the phenotype of virgin female mice did not progress beyond what appears to be accelerated growth, multiparous mice harbouring the Shc transgene displayed extensive epithelial hyperplasias and routinely developed hyperplastic alveolar nodules (HANs) (Figure 3.3B). In two instances, these hyperplastic mammary glands developed into multifocal mammary tumours (Figure 3.4) which arose adjacent to

hyperplastic mammary epithelium. Measurement of Shc protein levels in normal and tumour tissue argues that protein levels as well as its level of phosphorylation are responsible for tumour formation. While the overexpression of Shc may be weakly oncogenic, the focal nature of the tumours, long latency and weak penetrance suggest that additional genetic events are required to transform the mammary epithelium. Because mice did not develop hyperplasias until they had undergone multiple pregnancies, perhaps there is an additive effect of the transgene after subsequent litters. This effect may be due to the temporal pattern of transgene expression with respect to mammary gland differentiation. The MMTV LTR maintains low levels of transcription throughout the hormonally-induced morphogenic changes of the mammary gland with levels peaking at parturition (Hagar, 1988). Because the multiparous mice in this study had delivered between four and six litters, it will be necessary to determine the effect of each successive pregnancy on the relative Shc protein levels.

Another result of overexpressing Shc was that multiparous mammary glands did not undergo complete regression after weaning (Figure 3.3D). Interestingly, these mice did not demonstrate an inability to lactate. In wildtype mice, involution of the mammary gland is complete by 14 days after weaning (Li *et al.*, 1994), at which point the gland resembles a mature virgin mammary gland. The lactating gland, which is composed largely of epithelium responsible for the secretion of milk proteins, regresses to a quiescent organ composed predominately of fat cells surrounding a mammary epithelial tree. After four to six pregnancies, the mammary glands of these mice had developed hyperplasias.

Normal development and maintenance of tissue size is dependent upon a balance between cellular proliferation and apoptosis. The involution of secretory epithelium following pregnancy and lactation is the most dramatic example of apoptosis in mammary gland development. It has previously been demonstrated that the inhibition of apoptosis contributes to neoplasia (Andres, *et al.*, 1991; Raff, 1992). There is evidence to suggest that Shc may play a role in the suppression of apoptosis by inducing *myc* (Gotoh, *et al.*, 1996; Gotoh, *et al.*, 1997). However, it has recently been demonstrated that Shc mediates immediate-early gene expression and cell cycle progression by coupling $\alpha_6\beta_4$ integrins to Ras-Erk and Rac-Jnk pathways (Mainiero *et al.*, 1997). To assess whether the levels of apoptosis within the mammary glands of these Shc transgenic mice are normal, TUNEL assays should be performed throughout different stages and numbers of pregnancies.

4.3 Overexpression of Shc accelerates tumourigenesis in NDL and PyV MT backgrounds

The role of overexpression of Shc and its interaction with two oncogenes was explored by deriving bigenic mice. p52 Shc was overexpressed with an activated form of the Neu receptor tyrosine kinase (NDL) as well as with a middle T (MT) mutant (MT Y250F) debilitated in its capacity to bind and phosphorylate Shc. Many of the signaling pathways activated by Neu are also activated by MT, suggesting a common underlying mechanism of transformation. Neu receptor kinase activation effects redundant but equally effective means to activate the GDP/GTP-binding protein. Tyrosine residues

#1144 and #1226/7 become phosphorylated in the activated Neu receptor tyrosine kinase and form binding sites for Shc and Grb2 respectively. Both of these proteins effectively couple proteins responsible for Ras localization to the membrane and activation (Dankort *et al.*, 1997). MT shares the ability of Neu to bind Shc which in turn recruits the Ras-activating Grb2:SOS complex (Egan *et al.* 1993; Rozakis-Adcock, *et al.*, 1992). The fact that activation of Ras by tyrosine kinases is a common occurrence, suggests that this process is of great significance in transducing proliferative signals to the nucleus.

We sought to determine whether the overexpression of Shc in an NDL background could increase signaling through Shc and consequently accelerate tumour kinetics. The 23% decrease in latency and increased penetrance of tumour formation suggests that overexpression of Shc is able to increase proliferative signaling. One caveat of this experiment is that the NDL1-2 line expresses the transgene later than does the Shc line (Siegel, *et al.*, unpublished observations). This means that the window in which the two transgenes are collaborating is small. To further investigate this combination of transgenes, Shc mice should be mated to other NDL strains.

Another important issue that remains to be addressed is whether other receptors are hypersensitive to growth factor signaling when Shc is overexpressed. Shc lies downstream of a variety of growth factor receptors (Rozakis-Adcock *et al.*, 1992; Prigent and Gullick, 1994; Segatto *et al.*, 1993). Perhaps certain receptors can be activated by the simple probability of downstream targets.

Deletion of tyrosine residue 250 in MT, the site of Shc binding, interferes with the ability of MT to transform mammary epithelial tissues in transgenic mice. In contrast to

the rapid and multifocal nature by which MT transforms, MT Y250F transgenic mice develop focal mammary tumours over an extended period of time (Webster *et al.*, submitted to Mol. Cell. Biol.). These studies suggest that because tumours arise in MT Y250F animals with reversions to wildtype in only 7% of mammary tumours, either an indirect activation of Ras or the use of Ras-independent pathways in mammary tumourigenesis occurs. However, metastatic progression in these animals appears dependent upon Ras activation. 50% of lung metastases analysed had reacquired the Shc binding site through somatic mutation of the transgene, suggesting that lack of metastases in some of these animals may be due to inability to recruit Ras-activating proteins to the MT complex. To determine whether tumour formation and subsequent metastases is dependent upon Ras activation through Shc, I interbred MT Y250F mutant transgenic mice with Shc transgenic mice in the hope of recapitulating the wildtype MT signal. Theoretically, overexpression of Shc could rescue the MT Y250F mutant and encourage reversion of the Shc-binding site and mimic wildtype MT transformation tumour kinetics.

Consistent with previous studies (Webster, *et al.*, 1997), MT Y250 F virgin females developed mammary carcinomas with a T_{50}=155 days. Bigenic mice did experience a 20% decrease in latency and developed tumours with T_{50}=125 days. This increase in kinetics did not recapitulate kinetics of WT MT T_{50}=53 days. While wildtype kinetics were not mimicked, overexpression of Shc was able to accelerate the time of tumour onset suggesting that Shc is an important mediator of the MT signal. While the quantitative differences were relatively small, qualitative differences were noted in the bigenic mice, particularly in the wholemount analyses (Figure 3.10). Further numbers of

bigenic animals and analyses of transgenes from mammary tumours and lung metastases from these animals should provide considerable insight into the MT-mediated process of transformation and transgene reversion. Injection of MT Y250F and Shc/MT Y250F cell lines into syngeneic mice is a quick and alternative means of comparing rates of tumour formation and rates of metastases among these two genotypes. Lung metastasis observations, although a small sample size, indicate that overexpression of Shc in this system was able to increase the propensity to metastasize to the lung. These results suggest that signaling through Shc is an important step in MT-mediated tumourigenesis and metastasis.

One explanation for the requirement of Shc is that it provides the Shc/Grb2/SOS complex necessary to signal through Ras. Alternatively, Shc binding may also be recruiting other pathways. A number of studies have focused on understanding how a single tyrosine kinase can regulate multiple responses. The role of Shc may serve to facilitate or amplify signals. In PC12 cells, different levels and durations of MAPK activation lead to different cell fate outcomes (Traverse *et al.*, 1994). Rescue of a transformed phenotype in Rat1 cells containing a mutated Grb2 unable to bind BCR-ABL demonstrates that Shc overexpression functions to increase the dosage of Ras signaling in the absence of a direct BCR-ABL/Grb2 interaction (Goga *et al.*, 1995).

During carcinogenesis, the invasive growth of tumours is characterised by the penetration of the basement membrane and stromal invasion. Post-lactational involution of the mammary gland is also controlled by the expression of extracellular-matrix-degrading enzymes and their inhibitors. Because a small number of lung metastases from

the Shc/MT Y250F bigenic strain resembled the tumour phenotype of stromelysin transgenic mice and multiparous Shc transgenics the lack of tissue reconstruction in post-lactational mammary glands, it will be of value to investigate the expression of this matrix metalloproteinase in conjunction with overexpression of Shc.

An interesting observation of the Shc/MT Y250F lung metastases is that some of these metastases produced lipid droplets (Figure 3.11). This capability is not seen in either of the WT MT or MT Y250F metastases. It will be important to determine whether these lipid droplets are specific to milk. Analysis of a differentiation specifc gene such as beta-casein would accomplish this.

Shc/MT Y250F transgenic results mimic human pathologies. The propensity to metastasize to the lung mirrors one of the major sites of human metastases. The studies which have been described here provides insight into the involvement of the adaptor molecular Shc in the development and transformation of mammary epithelial tissues, both alone and in concert with other oncogenes. Similarities can be drawn between the events necessary to induce MT-mediated transformation and those important for receptor-mediated cell growth and transformation. MT effects transformation by manipulating apoptotic and proliferative responses simultaneously (Webster, *et al.*, manuscript in preparation) while Neu-mediated transformation requires the heterodimerization with family members to achieve similar results (Pinkas-Kramarski *et al.*, 1996). Interestingly, the adaptor molecule Shc appears to be common and important to both signaling pathways.

Further biochemical and molecular analyses of these bigenic mice will be instructive as to the type of secondary changes selected for *in vivo*. It is conceivable to think that the combinations of oncogenes studied in this thesis which appear to be cooperating, could be acting in different pathways, rather than forming part of the same linear pathway. It is possible that the synergism of Shc with NDL or MT Y250F could be strengthening one single pathway but this seems unlikely considering the number of molecules with which Shc is able to interact. The identification of the pathways involved may result in the development of therapeutic drugs designed to exploit these pathways, which could have important implications in the treatment of human breast cancer.

4.4 A model for Shc in cell proliferation and cell survival

Tumourigenesis is believed to occur when there is an imbalance in the actions of tumour suppressor genes and proto-oncogenes. Neu and MT share many of the same signaling pathways. One molecule common to these oncogenes is the adaptor molecule Shc. While both of these oncogenes depend upon the activation of Ras resulting in a proliferative signal through Shc and/or Shc:Grb2:SOS, the overexpression of Shc in multiparous animals suggests that Shc may also be involved in an anti-apoptotic pathway. Recent evidence suggests that proliferative signals are intimately coupled to those of apoptosis (Harrington *et al.*, 1994). While the oncogenic potential of Neu and MT appears to be the delivery of a strong proliferative signal, this does not appear to be the case for overexpression of Shc. Interruption of an apoptotic signal combined with an

enhanced proliferative signal could disrupt the delicate balance of a normal epithelial cell resulting in transformation or hyperplasia.

It is generally accepted that the activation of Ras plays a key role in triggering the cascade of events leading to cell proliferation. Because Shc has been shown to signal through Ras, it is of no surprise that overexpression of Shc could enhance growth in response to growth factors. Signaling pathways activated through Shc may affect multiple biological responses dependent on cell types. In most cases, tyrosine phosphorylation of the Shc adaptor protein promotes the formation of a complex of Shc and Grb2 at phosphorylated tyrosine residue 317-hShc/313-mShc. Grb2 is another SH2-containing adaptor protein which binds stably to mSOS, a Ras GDP/GTP exchange protein (Pawson, 1995). This pathway is implicated in Ras/mitogen-activated protein kinase (MAPK) activation towards c-*fos* gene expression. It has recently been reported that phosphorylation of Y317 of Shc is important for activation of Ras not only in growth factor signaling but G-protein coupled receptor or integrin receptor signaling (Wary *et al.*, 1996).

It now appears that Shc is able to amplify signals other than those mediated by Ras. Recent studies have identified novel tyrosine phosphorylation sites of Shc. Tyrosine 239/240 appears to have a previously unrecognised role in the induction of c-*myc* upon EGF stimulation, thereby suppressing apoptosis (Gotoh *et al.*, 1996). Upon stimulation with interleukin three in a hematopoeitic cell line, Shc becomes phosphorylated at three different residues. This signaling pathway appears to be distinct from the Ras/MAPK pathway. Again, phosphorylation of Y239/240 appears to play a

role in anti-apoptosis (Gotoh *et al.*, 1997). Involvement of other signaling pathways means that tyrosine-phosphorylated Shc may activate distinct pathways simultaneously. These phosphorylation sites of Shc appear to be evolutionarily conserved as the *Drosophila* Shc homologue conserves Y239/240 but not Y317 (Lai *et al.*, 1995). The recent identification of inositol polyphosphate-5-phosphatase Ship as a Shc-associating protein (Lioubin *et al.*, 1996) suggests that further dissection of Shc signaling is necessary to fully understand its role in mammary tumourigenesis.

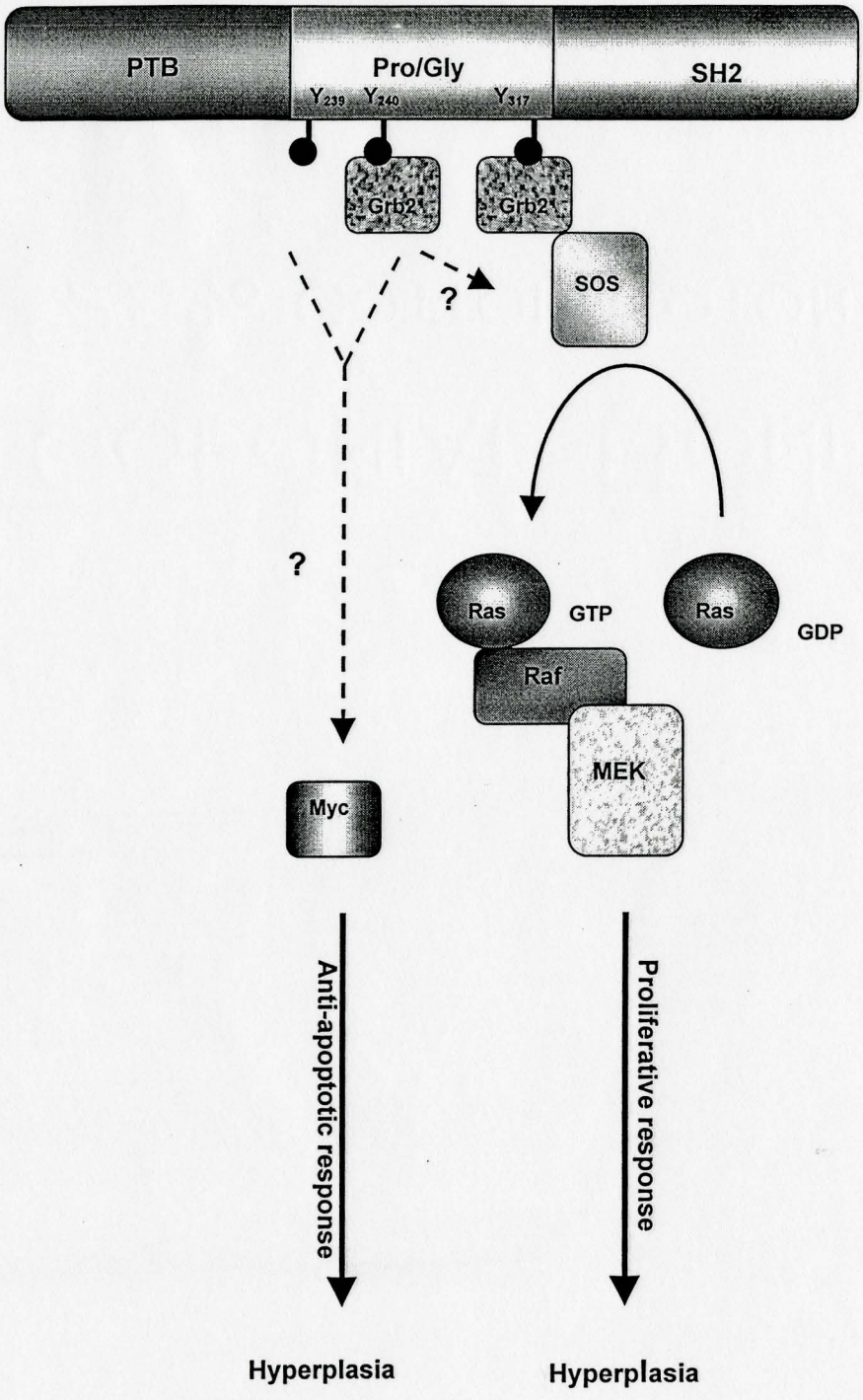
The MET receptor provides another example of Shc evoking multiple biological responses. Shc appears to act as an amplifier of a mitogenic as well as a motogenic response (Pelicci *et al.*, 1995a). The biological mechanisms responsible for the stimulation of motility and cell matrix invasion involves modification of cytoskeletal architecture and requires specific genes which encode proteolytic enzymes.

Shc expression alone is not sufficient to induce malignant conversion of the mammary epithelium but it does appear to be capable of altering mammary gland development. With other influences such as expression of other oncogenes and hormonal influences, Shc is able to evoke unique phenotypes. The evidence provided in this thesis supports the idea that Shc is able to provide proliferative signals as well as a cell survival signal (Figure 4.1). In general, there is little reason to believe that *in vivo*, a single oncogene can generate tumourigenic cells (see Weinberg, 1989 for review). The lack of adequate involution in the multiparous mammary glands may be the result of an anti-apoptotic signal. It is feasible that the overexpression of Shc and the consequent phosphorylation, may contribute to this activity.

The animal models which I have generated will be valuable in elucidating the molecular mechanisms of Shc. Identification of the molecules signaling through Shc will provide further insight into growth factor-induced signal transduction and its relation to tumourigenesis. It is likely that other effectors of Shc will be identified in the near future now that other binding sites on Shc have been identified (Gotoh *et al.*, 1997; van der Geer *et al.*, 1996). The identification of the molecular mechanisms by which Shc may alter/affect other oncogenes will provide important insight into the molecular basis of breast cancer.

Figure 4.1 A model for Shc in mammary development and tumourigenesis

Schematic representation of Shc with cellular associating proteins and their hypothetical biological effect. Hypothetical signal effects are represented as a broken arrow. The cellular fate of cells expressing the indicated Shc molecule is shown below the arrows. Other associating cellular proteins are omitted for clarity.


PTB
Pro/Gly
Y239 Y240
Y317
SH2
Grb2
Grb2
SOS
?
?
Ras
GTP
Ras
GDP
Raf
MEK
Myc
Anti-apoptotic response
Proliferative response
Hyperplasia
Hyperplasia

Chapter 5

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