NOTE TO USERS

THE ROLE OF β1-INTEGRIN RECEPTOR SIGNALING IN MAMMARY GLAND TUMOURIGENESIS

by

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ABSTRACT

Experimental and clinical evidence implicate the β1-integrin subunit and its associated intracellular effector ILK in the initiation and progression of human breast cancer. Roles for these proteins in promoting the oncogenic process, however, have not been demonstrated *in vivo*. As a result, experiments were designed to test the tumour-promoting properties of β1-integrin and ILK in the mammary glands of transgenic mice.

The first of these experiments involved the targeted ablation of a conditional allele of β1-integrin in a transgenic mouse model of human breast cancer. Using this approach, it was found that the expression of β1-integrin is required for oncogene-induced transformation of the mammary gland epithelium *in vivo*. This requirement for β1-integrin expression was observed during both the initiation of tumourigenesis, as well as for maintaining the growth of established tumours. In addition, a block in tumour cell proliferation following ablation of β1-integrin expression was found to be associated with the suppression of FAK autophosphorylation, providing a molecular mechanism underlying the requirement for β1-integrin expression during tumourigenesis.

The second experiment was designed to test the oncogenic properties of ILK, through the establishment of transgenic mice overexpressing ILK in the mammary gland epithelium. Following the induction of a hyperplastic mammary gland phenotype, these mice developed solid mammary tumours showing evidence of epithelial-to-mesenchymal transition, confirming that ILK overexpression can contribute to mammary tumourigenesis *in vivo*. Since expression of both β1-integrin and ILK have been reported in samples of aggressive human tumours, these results may have clinical significance to the treatment of human breast malignancies.

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A special thanks to Tom Rand, who played a big part in getting me here, as well as showing me the virtues of sound judgement and moderation. Let's hit the woods again

soon, brother, but this time I get to carry the knife. And by the way, I beat you to it, Mr. Rand.

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To my Mom and Dad, you can finally stop wondering if and when I was actually going to graduate. You deserve to enjoy this moment and share in this degree as much as I do.

And to my lifelong, childhood friends—

I FINALLY FINISHED SCHOOL!

(.....for now).

CONTRIBUTIONS BY OTHERS

Tumour cell transplants were performed under the skilled supervision of Natasza Kurpios, who not only taught me the technique, but performed more than her share of the work.

Immunohistochemistry for cytokeratin-8, E-cadherin and smooth muscle actin in MMTV/ILK-derived tumors (Figure 4.2.5.1) was performed by Judy Walls at the Center for Comparative Medicine, University of California at Davis, Davis, California.

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Mice harbouring the LoxP1-flanked alleles of ILK and β1-integrin were generated in the laboratories of Dr. Rene St. Arnoud and Dr. Ullrich Mueller, respectively, who kindly provided us with the animals.

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LIST OF ABBREVIATIONS

Ad	Adenovirus
BM	Basement Membrane
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMT	Epithelial-to-Mesenchymal Transition
ERK	Extracellular Signal Regulated Kinase
FAK	Focal Adhesion Kinase
GSK-3β	Glycogen Synthase Kinase-3 Beta
IB	Immunoblot
ILK	Integrin Linked Kinase
LTR	Long Terminal Repeat
MAPK	Mitogen Activated Protein Kinase
MMTV	Mouse Mammary Tumour Virus
MT	Middle Tumour antigen
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3-K	Phosphatidylinositol 3-Kinase
PKB	Protein Kinase B
PyV	Polyomavirus
RT	Reverse Transcription
SH2	Src Homology 2
SMA	Smooth Muscle Actin

CHAPTER 1

Introduction

1.1 The mouse as a model of human breast cancer

Breast cancer was the leading reported cause of cancer-related deaths among Canadian women in recent years, surpassing even those due to lung cancer (Table 1.1). These deaths represent almost 28% of total cancer-related deaths among women during this period. While surgical intervention generally remains the most effective method for the acute treatment of this disease, there remain problems associated with lymph node involvement and relapse. In addition, recent (albeit controversial) studies have called into question the relevance of early detection as a criteria for successful treatment (Freedman et al., 2004; Gotzsche and Olsen, 2000; Olsen and Gotzsche, 2001). For managing this disease in the future, therefore, it will be necessary to elucidate the environment and biological basis of the disease, including the nature of the underlying genetic lesions which contribute to the growth and dissemination of the tumours. By doing so, effective pharmacological strategies can be developed which may be used alone or in conjunction with traditional surgical, radiological and chemotherapeutic modalities.

The last two decades have seen some important progress in this regard. The discovery of the BRCA1 and BRCA2 genes, for example, have greatly increased our understanding of the etiology of familial or heritable forms of breast cancer (Casey et al., 1993; Welcsh and King, 2001; Welcsh et al., 1998). Similarly, the role of tumour suppressor gene products during disease progression has generated a great deal of interest, given that genes such as that for p53 have been found to be altered in a large proportion of human breast cancers (Elledge and Allred, 1994). In addition to tumour suppressor genes, molecules such as the HER2 receptor tyrosine kinase have emerged over the last twenty years as important prognostic indicators, implicated in promoting the tumour phenotype. Defining the roles of molecules such as HER2, p53 and BRCA1/2 in the etiology and progression of human breast cancers may eventually facilitate the

TABLE 1.1 Cancer Mortality Rates for 1997 (Canada)

Cancer	Deaths	Rate per 100,000
Buccal Cavity and Pharynx	70	0.9
Oesophagus	28	0.4
Stomach	113	1.5
Rectum	85	1.1
Intestine	442	5.9
Pancreas	175	2.3
Larynx	16	0.2
Lung	1207	16
Breast	1575	20.9
Cervix uteri	207	2.7
Thyroid	13	0.2
Hodgkin disease	24	0.3
Leukaemia	143	1.9
All cancers	5653	75.1
Bladder	29	0.4
Melanoma of skin	86	1.1
Gallbladder	38	0.5
Kidney	65	0.9
Non-Hodgkin lymphoma	202	2.7
Multiple myeloma, etc.	51	0.7

(Taken from the WHO mortality database, available online at http://www-depdb.iarc.fr/who/menu.htm)

development of effective treatment or screening protocols, to assist in the management of this disease. In the case of HER2, the discovery of the tumourigenic role for this protein has already led to attempts to develop targeted, antibody-based therapies against human breast cancer.

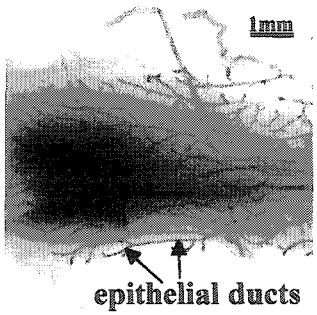
While most cancer-related genes and gene products were identified through genetic analysis of target populations and analysis of clinical samples, the relevance of these molecules to disease progression and their validation as drug targets was established primarily through the use of genetically modified mouse models. The oncogenic role for the HER2 receptor, for example, was ultimately confirmed following overexpression of a rat homologue of this molecule in the mammary glands of transgenic mice (Muller et al., 1988) (The rat and mouse homologues of HER2 are refered to as neu and erbB2, respectively, and are often used interchangeably). Prior to this experiment, the HER2 gene had been found to be overexpressed in samples of human breast cancer (Slamon et al., 1987), and had satisfied the criteria of an oncogene in cultured mammalian epithelial cells. Strategies to express a putative oncogene such as HER2 in a mouse tissue *in vivo* therefore provided researchers with a powerful tool to elucidate the specific contribution of these molecules to the pathological state, in a physiologically relevant context (Figure 1.1.1). Many of the molecules which have been evaluated using this transgenic approach are listed in Figure 1.1.2. In addition, a survey of genetically modified mice used as models to study the initiation and progession of human breast cancer is presented in several comprehensive reviews (Cardiff et al., 2000; Hutchinson and Muller, 2000; Siegel et al., 2000).

The utility of mouse models as an experimental tool lies in the ability to cross the various strains, in order to further dissect the molecular genetic basis of breast cancer *in vivo* (Figure 1.1.1). Such crosses have provided numerous examples of genetic lesions which are needed to co-operate for the efficient induction of mammary gland tumourigenesis. Expression of the small GTPase Ras under a mammary-specific promoter, for example, results in the induction of focal mammary tumours at an average

Figure 1.1.1 Evaluating the roles of human breast cancer-associated genes *in vivo*

The oncogenic potential of a putative human oncogene can be tested *in vivo* by directing expression of the gene to the tissue of a mouse, such as the mammary gland epithelium (ie. GENETIC LESION #1). The role of co-operating oncogenic events or tumour suppressor genes (ie. GENETIC LESION #2) can then be determined by crossing various mouse models, and evaluating the impact on mammary gland transformation and tumourigenesis. Oncogenic events are indicated by the presence of neoplastic mammary gland lesions (green arrows in lower left panel), or solid mammary tumours (lower right panel). The top panel shows a whole mount of a normal mouse mammary gland, prior to transformation.

Normal mouse mammary gland epithelium:



GENETIC LESION #1
putative human oncogene such as HER2(erbB2)

Genetic cross through animal breeding

GENETIC LESION #2
co-operating oncogene, dominant negative allele, germline or targeted gene deletion (eg. loss of tumour suppressor)

Oncogenically transformed mammary gland epithelium:

Mammary gland tumour

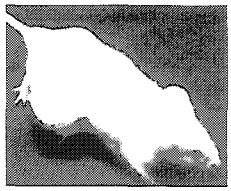


Figure 1.1.2 The physiological role of numerous cancer-associated genes have been tested in genetically modified mouse models

Listed are the genes which have been overexpressed in the mouse mammary gland epithelium, in order to evaluate their biological potential as oncogenes. The expression of most of these genes was directed to the mammary gland epithelium using either the mammary-specific mouse mammary tumour virus (MMTV) long terminal repeat (LTR), or the whey acidic protein (WAP) promoter. The role that these proteins play in normal cellular physiology is included beside the list. Also indicated are the genetic crosses which have been performed between various mouse models, involving either two transgenes (eg. *Ras* x *c-myc*) or a transgene and a targeted gene deletion (eg. *c-myc* and $p53^{-/-}$). Note that whereas most of these genes represent human cellular oncogenes and tumour suppressors, the middle tumour (mT) antigen of polyomavirus (PyV) has also been used to oncogenically transform the mouse mammary gland. The PyV mT antigen has commonly been used as a surrogate for human oncogenes, as will be discussed in a later section.

Growth factors	*FGF*-3 (*int*-2)
	FGF-7 (kgf)
	FGF-8
	MGF
	NDF
	SGF
	TGFα
	TGFα X c-*myc*
	TGFαX *neu*
	TGFαX Stat5a$^{-/-}$
	*TGF*b2
Receptors	
	ErbB2/neu
	ErbB2/neu X TGFα
	Met
	Ret
Differentiation	
	Notch (*int*-3)
	Wnt 1 (*int*-1)
	Wnt 1 × *int*-2
	Wnt 1 × Era$^{-/-}$
	Wnt 1 × VP
	Wnt 10b
Signal transduction	
	PyV-mT
	PyV-mT × *Ets2*$^{+/-}$
	PyV-mT × *Grb2*
	PyV-mT × *Shc*
	PyV-mT × *src*$^{-/-}$
	PyV-mT × yes$^{-/-}$
	Ras
	Ras × c-*myc*
	Src
	PTPe
Cell-cycle	
	Myc
	Myc × p53$^{-/-}$
	Myc × TGFα
	Myc × *ras*
	Cyclin A
	Cyclin A × cdk2
	Cyclin D1
	Cyclin E
	SV40-Tag
	SV40-Tag × Bax$^{-/-}$
	SV40-Tag × p53$^{-/-}$
Other	
	Int 5 (aromatase)
	Str 1

(From Cardiff et al. (2000). References accompanying each strain are included in the original manuscript).

age of 5 to 6 months, while overexpression of the c-myc transcription factor results in tumours at just under 1 year of age (Sinn et al., 1987). Introduction of both of these transgenes into the same mouse, however, accelerates the onset of tumourigenesis to 46 days, demonstrating the co-operative effect of these molecules during mammary tumour induction (Sinn et al., 1987).

The experimental value of mouse models has also been demonstrated through the use of genetic strategies designed to ablate expression or activity of a gene *in vivo*. The contribution of the Ets family transcription factor PEA3 in erbB2/neu-mediated mammary tumour progression has been demonstrated using this approach. In this case, expression of a dominant-negative PEA3 allele dramatically delayed the onset of tumourigenesis in the erbB2/neu mammary tumour model (Shepherd et al., 2001). Similarly, this approach has been used to confirm the tumour suppressor roles of gene products such as the p53 protein. By crossing mice expressing oncogenes such as erbB2/neu with those harbouring inactivating mutations in the p53 gene, the important physiological role that this tumour suppressor plays during tumour progression has been validated in a live animal model (Li et al., 1997a; Zelazny et al., 2001).

The role of proteins such as p53 has also been examined through the targeted deletion of the respective gene sequence from the genome of mouse tumour models (McCormack et al., 1998). The deletion of many genes from the mouse germline, however, has been found to be associated with developmental abnormalities, including embryonic lethality. For example, mice harbouring a partial deletion in the BRCA1 gene sequence die at an early embryonic state when the deletion is present in the homozygous state (Liu et al., 1996). As a result, novel genetic approaches have recently been introduced in order to facilitate excision of gene sequences specifically in adult target tissues (Sauer, 1998). The embryonic lethality of BRCA1 mutations, for example, was overcome by targeting the excision of BRCA1 sequences specifically in the mammary epithelium of adult mice (Xu et al., 1999b). Following targeted ablation of the BRCA1 sequence, these mice developed mammary tumours characterized by massive genomic instability. This tissue-specific gene targeting approach confirmed that the BRCA1 gene

product plays an important role in DNA repair and genomic integrity *in vivo*, which otherwise could not have been determined through traditional germline gene targeting approaches.

The molecules described above--BRCA1/2, p53, erbB2 etc.--were initially found to be overexpressed, mutated or absent in human cancers, prior to their manipulation in the mouse. The use of transgenic and gene targeting strategies provided a means by which the role of these genes in disease progression could be examined in a physiological context. (The work presented in the Results section of this thesis uses both of these technologies in order to address the role of β1-integrin signaling in mammary gland tumourigenesis). Evaluating the role of tumour-associated molecules in the mouse, however, requires a biological phenotype which is readily discernable and reproducible. In the case of tumour-promoting mutations in the mouse mammary gland, for example, the mice would be examined for the appearance of solid mammary tumours, which can be easily accomplished by physical palpation. If the transgene or mutation is expected to result in a metastatic tumour phenotype, the lungs, liver or bone can subsequently be examined for the presence of metastatic lesions. In addition, the mammary glands of genetically modified mice can be examined for the presence of neoplastic and pre-neoplastic lesions, through the preparation of stained mammary gland whole-mounts and histological sections. Comparing these different phenotypic aspects between mice harbouring different combinations of transgenes and gene deletions provides an effective means to genetically dissect the roles of various tumour-associated mutations in tumour induction and progression.

In order to make reliable conclusions regarding the role of a gene in human breast cancer, however, it is necessary to first establish whether the murine phenotype resembles some aspect of human disease. As a result, mouse models of human breast cancer are often examined for the presence of morphological and pathological criteria to facilitate classification of the tumour phenotype (Cardiff, 2001; Cardiff et al., 2004). Ultimately, this classification will serve to facilitate comparative analysis between mouse models and human disease. By matching gene expression profiles with pathological outcomes in

both murine and human tumour samples, important and novel therapeutic targets and prognostic indicators can be identified. In addition, the genetically modified mice may provide reliable models for testing the efficacy of novel drugs *in vivo*.

Numerous strains of genetically modified mice are currently available as models of various aspects of human breast cancer progression (Cardiff et al., 2000; Hutchinson and Muller, 2000; Siegel et al., 2000). These include models of early pre-malignant lesions such as ductal carcinoma *in situ* (DCIS), various forms of adenocarcinomas, and models of metastatic disease. These models have proven to be valuable in elucidating the contribution of various genes to particular stages of neoplastic disease. Nonetheless, the need remains for the establishment of additional, novel strains which model some under-represented aspects of human cancers. For example, there are relatively few mouse models which recapitulate the phenomenon of epithelial-to-mesenchymal transition (EMT), in which transformed epithelial cells aquire the morphological characteristics of motile, fibroblast-like cells. Identifying genes which can contribute to EMT in the mouse would be valuable from a clinical perspective, since mesenchymal-like tumours are often very aggressive (Thiery, 2002).

The experimental results presented in this thesis describe both the establishment of a novel transgenic mouse model of mammary gland tumourigenesis, as well as the use of targeted gene ablation technology in order to address the role of β1-integrin signaling in an established mouse model of human breast cancer. The remaining sections of this introductory chapter, therefore, are intended to 1) provide background on the anatomy of the mouse mammary gland, and 2) discuss the ways in which the physiology of the gland is regulated by the concerted signaling properties of hormones, growth factor receptors, and cell adhesion events, specifically through the integrin family of cell adhesion receptors. The interplay of these molecules will be discussed as a preamble to the way in which deregulated receptor signaling can lead to oncogenic transformation of the mammary gland. A putative role for the β1-integrin molecule and its associated integrin-linked kinase (ILK) in human breast cancer will then be discussed with reference to both

clinical and experimental data, providing a rationale for the experiments described in the remainder of the thesis.

1.2 The murine mammary gland: setting the stage for disease

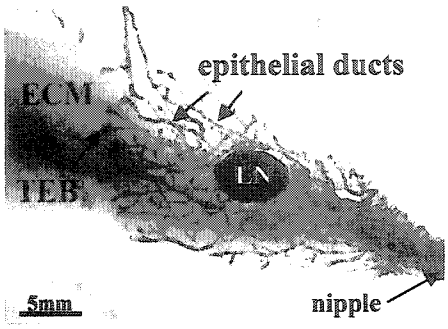
The murine mammary gland consists of an arbourized network of epithelial ducts, embedded within a stroma of adipose cells, fibroblasts, lymphatics, blood vessels, and an extracellular matrix (ECM) of fibrous proteins such as collagen (Figure 1.2A) (For excellent reviews on the structure and development of the murine mammary gland, see Hennighausen and Robinson (1998), Hovey et al. (2002), Li et al. (1997), Prince et al. (2002), Richert et al. (2000), and Streuli (2004)). Development of the mouse mammary gland begins *in utero* with the appearance of a primordial mammary bud derived from the primitive ectoderm. By the time of birth, the precursor gland has grown into a rudimentary mammary tree, exhibiting the first signs of a simple branching pattern. Growth of the gland stops temporarilly following the birth of the animal, and resumes at approximately 3 weeks post-partum, coinciding with the onset of puberty. A period of rapid growth then follows over the next several weeks, during which time the expanding mammary tree penetrates into the supporting fat pad, while undergoing a process of extensive lateral branching (Hennighausen and Robinson, 1998; Richert et al., 2000).

Growth of the mammary tree during puberty is due to massive proliferation in a progenitor population of cells residing in terminal end bud (TEB) structures at the leading end of each growing duct (Figure 1.2A). It is from these TEB structures that a process of bifurcation leads to the generation of new ducts, contributing to the extensive network present in the adult gland. By approximately 8 weeks of age, the expanding mammary tree has reached the perimeter of the fat pad, at which time there is cessation of growth, accompanied by the disappearance of the TEBs. At this point, which co-incides with the sexual maturity of the animal, the central lumen of each mammary duct is lined by two concentric layers of specialized epithelial cells—an inner layer of polarized luminal epithelial cells, which are immediately adjacent to the central lumen, and an outer layer of myoepithelial cells (Figure 1.2B). These epithelial layers, in turn, are supported by a

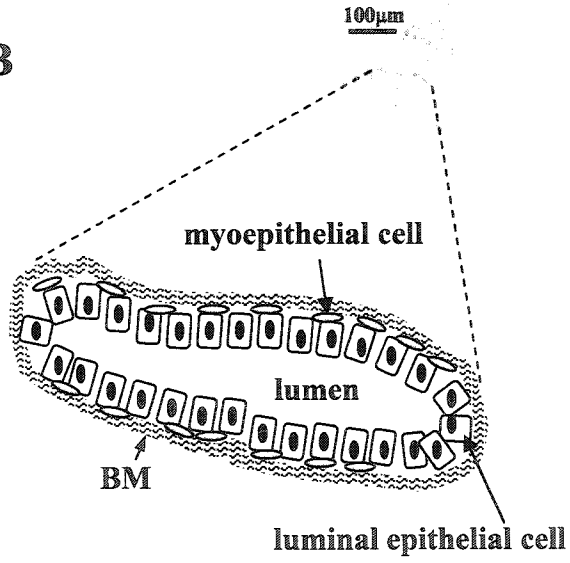
Figure 1.2 Anatomy of the mouse mammary gland

Shown in (A) is a mammary gland wholemount from a 6 week-old virgin female FVB mouse. At this age, the epithelial ducts have not yet filled the supporting mammary gland fat pad. Development of the gland through puberty is described in the text. LN designates the lymph node, used as a convenient marker for comparing the rate of ductal outgrowth between animals. (B) Orientation of the epithelial cell layers within a ductal cross section. BM designates the basement membrane, a specialized region of the extracellular matrix (ECM). (C) During pregnancy and lactation regions of the gland reorganize into milk-producing alveolar structures.

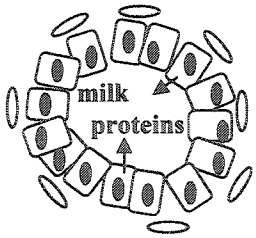
A


epithelial ducts
ECM
LN
TEB
5mm
nipple

B


100μm
myoepithelial cell
lumen
BM
luminal epithelial cell

C


milk
proteins

thin, specialized region of the ECM called the basal lamina or basement membrane (BM), which surrounds the entire ductal structure (Figure 1.2B). Both the luminal epithelial and myoepithelial cells of the adult gland associate with components of the BM (laminin-1, fibronection, collagen IV etc.) through specific adhesion receptors expressed on the cell surface (Prince et al., 2002; Streuli, 2004).

The architecture of the 'resting' adult mammary gland would be maintained throughout the life of the mouse if not for the parous nature of the animal. During mid to late stages of pregnancy the gland undergoes a process of differentiation in order to facilitate the production of milk for feeding the offspring (Hennighausen and Robinson, 1998). Preparation for milk production and delivery involves a morphological transformation of the gland, in which regions of the ductal epithelium are reorganized into spherical, milk-producing alveoli (Figure 1.2C). In the alveolar orientation, the luminal epithelial cells are surrounded by a sparse, contractile layer of myoepithelial cells, which are arranged in a loose lattice formation. Soluble milk proteins produced by the differentiated luminal epithelial cells accumulate in the central lumen of each alveolus, while contraction of the myoepithelial layer subsequently forces the milk into the subtending ducts. These milk-producing alveolar structures appear as the result of extensive side-branching and budding along the length of the mammary ducts. During this time, the overall mass of the glandular epithelium increases due to massive proliferation of the luminal epithelial cells. As a result, the epithelial proportion of the gland increases from approximately 10% in the virgin state to 80-90% during lactation. Following withdrawal of the pups, the epithelial population is reduced through programmed cell death, while the mammary tree and stromal elements undergo a process of remodeling in order to return to a structure resembling that prior to pregnancy (Li et al., 1997c).

A cycle of proliferation, differentiation and programmed cell death therefore underlies the morphological and functional development of the murine mammary gland. As a result, preserving the normal physiology of the gland requires maintaining a balance between the timing and duration of these processes during the life of the animal. Indeed,

pathologies of the human mammary gland, such as hyperproliferative disorders of the breast, are now regarded as manifestations of alterations in this balance.

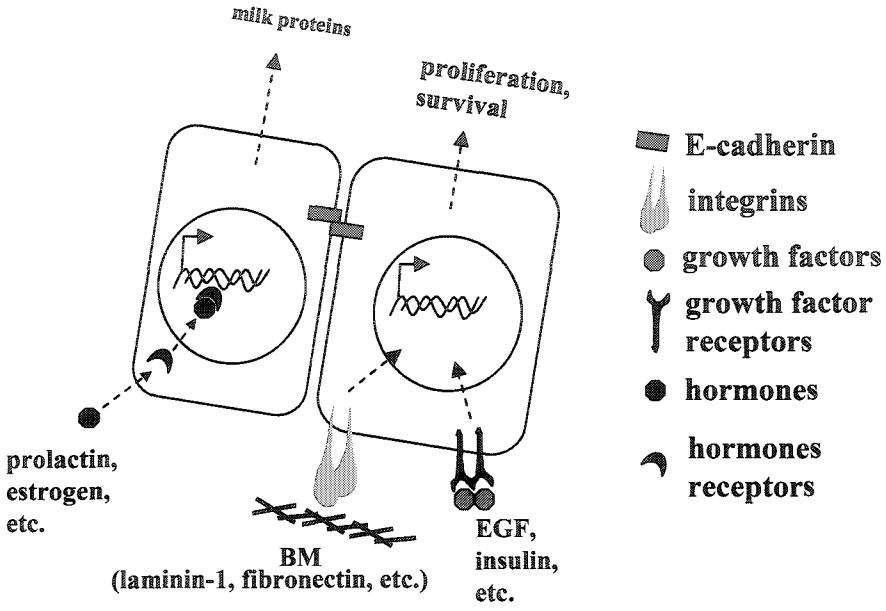
1.3 Hormones and growth factors regulate mammary gland structure and function

Development of the murine mammary gland, from early post-natal stages through puberty and into pregnancy, requires the co-ordinated action of numerous endocrine hormones and growth factors such as estrogen, prolactin, epidermal growth factor (EGF) and insulin (Hovey et al., 2002). Much of the knowledge regarding the roles of these molecules in mammary gland physiology has been provided by a combination of animal studies and the manipulation of cultured mammary epithelial cells (Hovey et al., 2002; Shillingford and Hennighausen, 2001). In the early days of this research, experiments using live animals often involved surgical or pharmacological techniques, such as exposing the role of estrogens in mammary gland development through removal of the ovaries (Flux, 1954). More recently, gene 'knock out' and tissue-specific gene targeting strategies have expanded our understanding of the developmental and physiological roles of many of these molecules (Hennighausen and Robinson, 2001; Kelly et al., 2002; Shillingford and Hennighausen, 2001).

The overall response of the mammary gland tissue to these hormones and growth factors ultimately depends on the concerted response of the individual cells populating the gland. When a diffusible hormone or growth factor reaches a target cell within the mammary epithelium, for example, the fate of the cell and perhaps the tissue will depend on whether that cell has been instructed to proliferate, differentiate or die. In most cases this response originates from the cell nucleus (Figure 1.3). For hormones such as estrogen, the activation of gene transcription is facilitated by a relatively simple process in which a complex between the hormone and an intracellular receptor serve directly as a DNA-binding transcriptional regulator (Hall and McDonnell, 1999). For growth factors such as EGF, however, the transmission of a signal through several intracellular effector molecules is often necessary to produce a nuclear response (Hunter, 2000). Binding of EGF to the membrane-bound EGF receptor (EGFR), for example, results in

Figure 1.3 The growth properties of luminal epithelial cells are regulated by a variety of receptors and ligand-induced signals

The differentiation, proliferation and survival of luminal epithelial cells are controlled by the co-ordinated action of growth factors, hormones and cell adhesion events. The concerted response of individual epithelial cells ultimately dictates the developmental state of the entire tissue.


milk proteins
proliferation, survival
E-cadherin
integrins
growth factors
growth factor receptors
hormones
hormones receptors
prolactin, estrogen, etc.
BM (laminin-1, fibronectin, etc.)
EGF, insulin, etc.

phosphorylation of the cytoplasmic domain of the receptor, providing binding sites for a variety of signaling molecules, such as the adaptor proteins Grb2 and Shc (Schlessinger, 2000). If mitogenic effectors such as the small GTPase Ras are subsequently activated by the binding of these molecules, the cell may respond by proliferating (Basu et al., 1994). Cell division in this case would likely involve the activation of downstream targets such as the mitogen activated protein kinase/extracellular regulated kinase (MAPK/ERK) (Basu et al., 1994; Hunter, 2000). Similarly, binding of the lipid kinase phosphatidylinositol 3-kinase (PI3-K) to the insulin receptor might result in sustained cell survival signals induced by the binding of insulin (Delcommenne et al., 1998; Downward, 2004). Tight regulation of these pathways is therefore critical for maintaining the integrity of the mammary gland. Transgenic mouse models of human breast cancer have indeed provided convincing demonstrations of the pathological outcomes when some of these signaling processes are deregulated (Hutchinson and Muller, 2000; Siegel et al., 2000).

1.4 The biological effects of growth factor and hormone signaling are modulated by adhesion to the basement membrane

The structural and functional integrity of the mammary gland, however, is believed to depend as much on the presence of the BM as on the diffusible hormones and growth factors (Figure 1.3). This dependency on the BM has been demonstrated almost entirely in cell culture-based experiments, where adhesion of primary mammary epithelial cells to an underlying substratum was shown to be necessary for the biological response to exogenously added growth factors and hormones (Deugnier et al., 1999; Lee and Streuli, 1999; Streuli et al., 1995). For example, adhesion of primary mammary epithelial cells to an artificially reconstituted BM has been shown to be necessary for the production of milk proteins (eg. casein), following the addition of the lactogenic hormone prolactin (Streuli et al., 1995). Similarly, the mitogenic response to EGF and the promotion of cell survival by exogenously added insulin require adhesion to BM molecules such as collagen or laminin (Deugnier et al., 1999; Farrelly et al., 1999; Lee

and Streuli, 1999). Forcing these otherwise adhesive mammary epithelial cells into suspension often results in attenuation of the cell growth or cell survival pathways induced by these growth factors (Farrelly et al., 1999), revealing an important clue regarding the molecular basis for this phenomenon (discussed in detail in Section 1.6 below).

In spite of being demonstrated primarily in cell culture models, the interdependency between cell adhesion and soluble hormones and growth factors is believed to reflect a physiological need for cells in a tissue to maintain a proper spatial orientation. Otherwise, proliferation occuring outside of an appropriate 3-dimensional context could dramatically alter the architecture and functional integrity of an epithelium, such as that of the mammary gland. A strict requirement for BM-derived survival signals, for example, may play an important role in protecting against the metastatic spread of mammary epithelial cells undergoing the early stages of transformation induced by activated growth factor receptors.

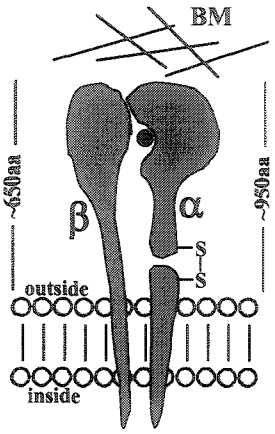
1.5 Molecular regulation of cell adhesion: the integrin family

Adhesion of mammary epithelial cells to the underlying BM is mediated primarily by the integrin family of transmembrane receptors. Integrin receptors are heterodimeric molecules, consisting of a single α subunit glycoprotein non-covalently associated with a single β subunit (Figure 1.5A). Each subunit consists of a large extracellular domain, a single-pass transmembrane domain, and a short cytoplasmic tail. Juxtaposition of the large amino-terminal domains of each subunit provides a binding site for components of the BM, such as laminin, fibronectin or vitronectin. In total, there are 18 known α subunits and 8 β subunits, which can dimerize to form up to 24 different integrin receptors (Figure 1.5B). Most integrin receptors typically have the capacity to bind more than one extracellular ligand. Conversely, each ligand may be recognized by more than just one integrin receptor. In addition to BM-derived ligands, a limited number of integrin

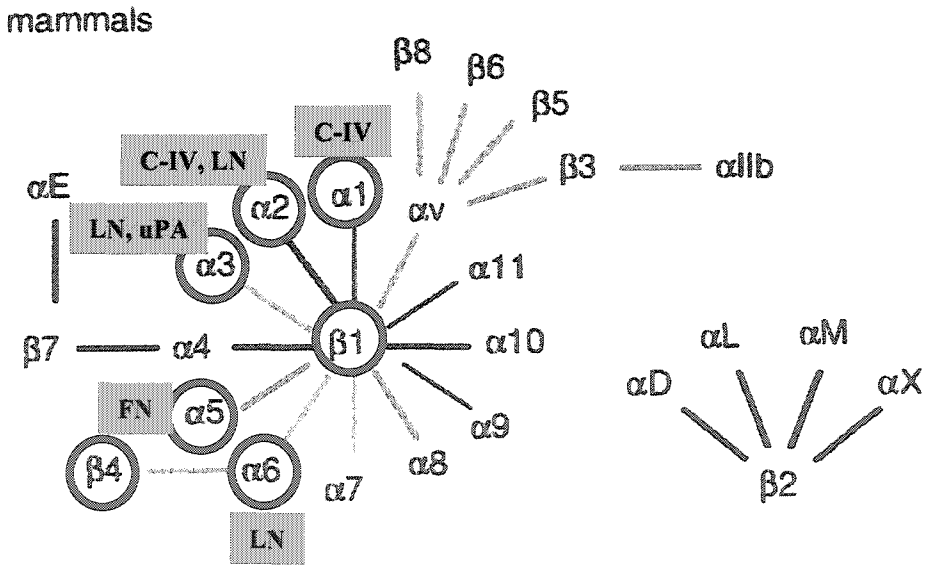
Figure 1.5 Adhesion to the basement membrane is mediated primarily by the integrin family of transmembrane receptors

(A) Basic structure of an integrin receptor, composed of a heterodimer of a single α and single β subunit. The large globular domain serves as a receptor for BM components such as collagen IV, fibronectin and laminin-1. The approximate sizes of the large extracellular domains are indicated. (B) A variety of α and β subunits combine to form up to 24 known integrin receptors in vertebrates. Subunits expressed in the mammary gland are circled in red, while their ligands appear in green rectangles. (Adapted from a figure in Danen and Sonnenberg (2003)).

A



B



from Danen *et al.* (2003) *J Pathol*, 200:471-480

heterodimers bind to other cell surface molecules, such as members of the immunoglobulin superfamily, where they mediate cell-cell interactions. The nature of integrin ligands, both BM-derived and membrane-bound, reflects the baso-lateral distribution of integrin expression within polarized epithelial cells (Taddei et al., 2003).

The repertoire of integrin receptors expressed within a tissue allows that tissue to respond to its particular extracellular environment. The mouse mammary gland, for example, is surrounded by a BM rich in laminin-1 and collagen IV, while the epithelial cells populating the gland express receptors for both of these ligands (Prince et al., 2002; Taddei et al., 2003) (Subunits expressed in the mammary gland are circled in red in Figure 1.5B, and known ligands for each αβ receptor pair are included in green rectangles). Given the diversity of tissue types in mammals, and the need for most of those tissues to interact with the immediate extracellular environment, it is no surprise that integrins are widely expressed throughout virtually all tissue types. The biological functions of these receptors are diverse, including roles in neurological physiology, bone remodelling, platelet aggregation and immune surveillance. As a result, integrins have been implicated in the etiology and progression of several human pathologies, such as leucocyte adhesion deficiency type 1 (LAD-1) (Wehrle-Haller and Imhof, 2003), congenital muscular dystrophy (CMD) (Mayer et al., 1997), demyelinating disease (Kanwar et al., 2000) and clotting disorders (Ruiz et al., 2001). In addition, the overexpression or activation of various integrin subunits and receptors have been associated with the progression and dissemination of various human cancers (Shaw, 1999; Wehrle-Haller and Imhof, 2003).

1.6 The biological roles of integrins are mediated by intracellular signaling complexes

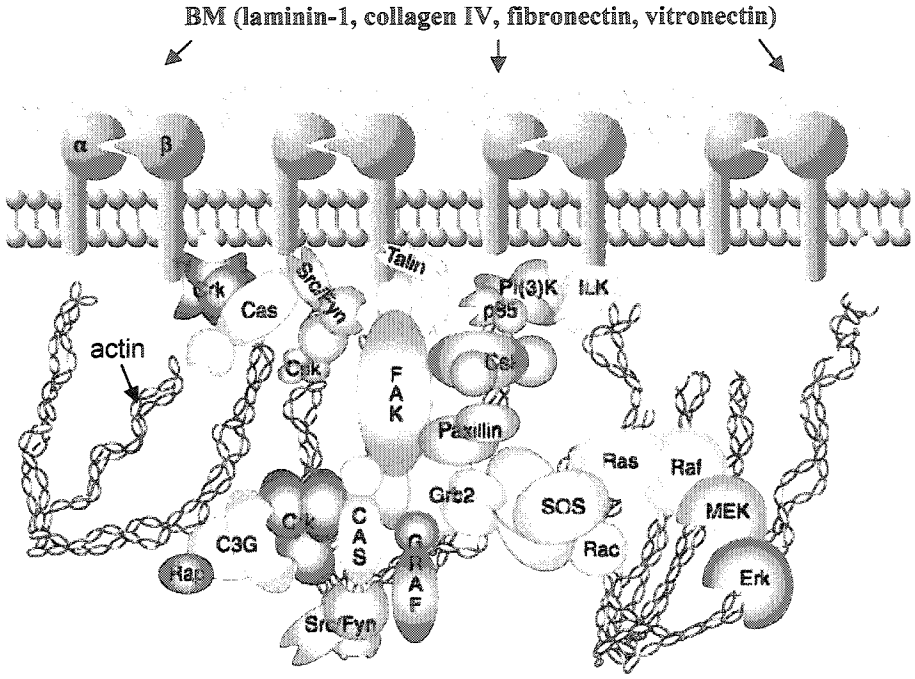
By binding to the extracellular domain of αβ heterodimers, BM constituents serve as ligands for the integrins, analogous to the relationship between growth factors and their cognate receptors. The occupancy of an integrin receptor by an extracellular ligand can result in a variety of biological responses, including cell spreading and migration,

proliferation, protection against apoptosis, and differentiation (Giancotti and Ruoslahti, 1999). Unlike most growth factor receptors, however, integrins lack intrinsic kinase activity and therefore do not undergo autophosphorylation upon ligand binding. Integrin-mediated biological responses, rather, are induced by the formation of large signaling complexes on the inner side of the cell membrane (Miranti and Brugge, 2002) (Figure 1.6). Molecules identified within these complexes include the c-Src family of tyrosine kinases, focal adhesion kinase (FAK), integrin linked kinase (ILK), PI3-K, Grb2, Shc, $p130^{CAS}$, Crk, paxillin and the Ras and Rho/Rac/cdc42 family of small GTPases. Some of these molecules, such as ILK, interact directly with the cytoplasmic domains of the β integrin subunits, whereas the majority associate indirectly by binding adaptor or scaffolding proteins such as paxillin. Overall, the repertoire of molecules in these complexes is diverse, facilitating the various cellular responses to integrin engagement.

By spanning the cell membrane, integrin receptors therefore provide a physical link between the BM and the intracellular signaling machinery. This property of integrin receptors provides a molecular mechanism underlying the requirement for cell adhesion in the maintenance of tissue homeostasis. Integrin receptors, however, also differ from growth factor receptors in that the extracellular binding properties are subject to regulation by intracellular signaling events (Giancotti and Ruoslahti, 1999; Hughes and Pfaff, 1998). Phosphorylation of the cytoplasmic tail by associated kinases, for example, can result in conformational alterations which are propagated to the extracellular ligand-binding domain of the integrin molecule (Caron, 2003; Kinashi et al., 2000). Such alterations may result in a change in the affinity of the receptor for its ligand. Similarly, re-organization of the actin-based cytoskeletal network, through the activation of molecules such as Rho, Rac or cdc42, can influence the degree of integrin clustering in the cell membrane, thus changing the avidity of an adhesion event. These processes are collectively refered to as 'inside-out' signaling, a phenomenon unique to integrin receptors (Giancotti and Ruoslahti, 1999).

Figure 1.6 Large intracellular signaling complexes form at the site of integrin clustering

The cytoplasmic domains of integrin receptors provide nucleation sites for the assembly of large signaling complexes, containing numerous kinases, phosphatases, scaffolding and adaptor proteins, and cytoskeletal proteins such as actin. The clustering of integrins at focal adhesion sites is mediated by both the basement membrane (BM) and the actin-based cytoskeleton. The formation of these signaling complexes mediates the cellular response to adhesion. (Adapted from a figure in Miranti and Brugge (2002)).



from Miranti and Brugge (2002) *Nature Cell Biol*, 4:83-90

1.7 Integrins and growth factor receptors collaborate through intermolecular cross talk

As discussed in Section 1.3, adhesion to BM components is necessary for the optimal induction of a cellular response to growth factors and hormones. In many cases, this interdependency has been shown to be the result of molecular cross talk between integrins and ligand-bound growth factor receptors such as those for insulin or EGF (Farrelly et al., 1999; Moro et al., 1998). This cross talk is often facilitated by the formation of adhesion complexes, where growth factor receptors and integrins are brought into close proximity. The cross talk may occur through the simultaneous binding of a membrane-proximal effector, such as ILK, or through the concerted activation of downstream targets, such as components of the MAPK pathway (Miyamoto et al., 1996). In addition, there is evidence of a direct reciprocal relationship between the expression and activity of integrin and growth factor receptor molecules themselves (Moro et al., 1998; Wang et al., 1998). Phosphorylation of the EGF, PDGF and FGF receptors, for example, has been shown to be induced by antibody-mediated activation of the β1-integrin subunit, resulting in synergistic activation of the MAPK pathway (Miyamoto et al., 1996; Moro et al., 1998). Similarly, overexpression of the EGF receptor in a human mammary cell line leads to upregulation of the β1-integrin subunit, while antibody-mediated inhibition of either receptor results in downregulation of both, as well as inhibition of MAPK signaling (Wang et al., 1998).

The biological importance of this molecular cross talk is perhaps best illustrated by the regulation of cell cycle progression in cultured cells, where the sustained activation of ERK, necessary for the G1 to S transition, requires both serum and integrin-mediated cell adhesion (Renshaw et al., 1997). Similarly, transcriptional upregulation of the cyclin D1 gene, as well as proteosomal degradation of the the p21Cip1 and p27Kip1 cell cycle inhibitors, require ligand-mediated occupation of the integrin receptors, as well as serum-derived growth factors (Assoian and Schwartz, 2001; Bao et al., 2002; Zhao et al., 2001). In almost all cases, integrin-associated FAK activity has been shown to be a critical determinant of integrin-mediated cell cycle progression and receptor cross talk

(Oktay et al., 1999; Renshaw et al., 1999; Sieg et al., 2000; Zhao et al., 2001). A model of integrin-growth factor receptor cross talk is presented in Figure 1.7.

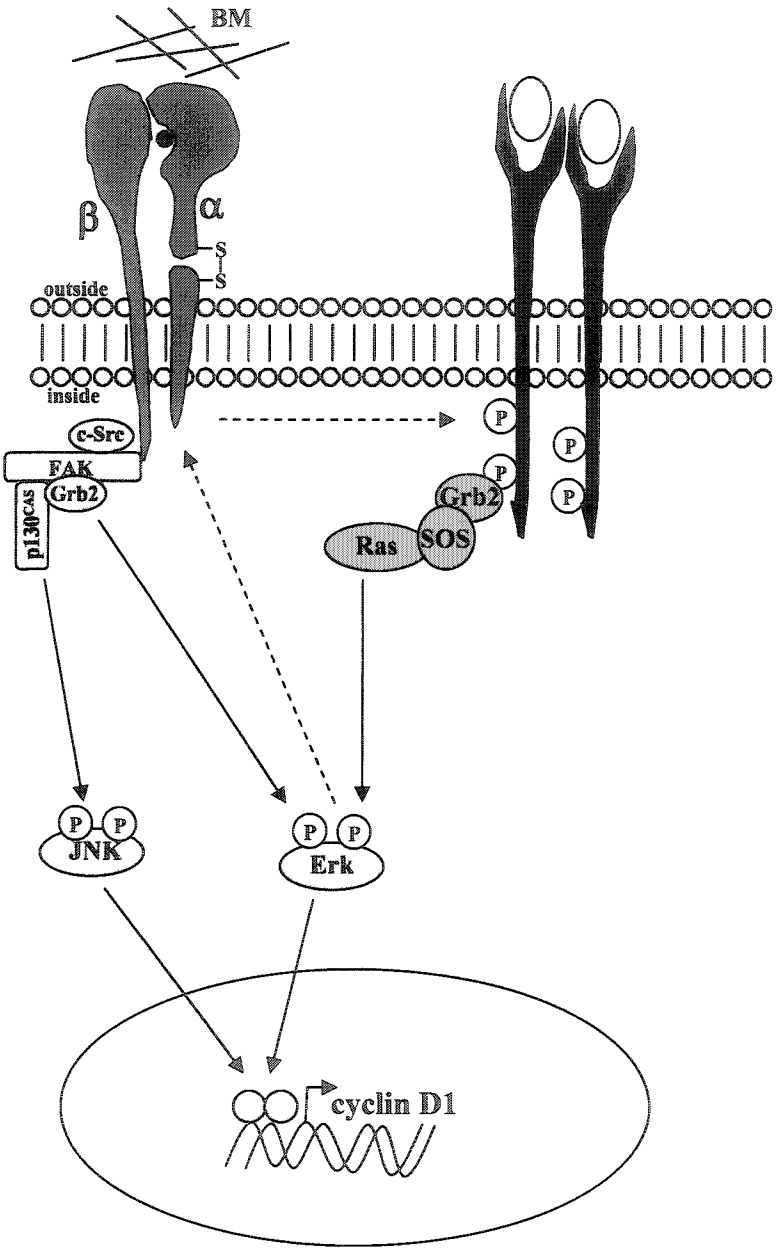
1.8 Cross talk between integrins and growth factor receptors may play an important role in mammary gland tumourigenesis

There is experimental evidence that the reciprocal signaling and cross talk between integrins and growth factor receptors may be critically important for promoting the progression of human breast cancer. Ligand-mediated induction of erbB2/erbB3 receptor signaling in human breast cancer cells, for example, results in modulation of β1-integrin binding properties (Adelsman et al., 1999). Similarly, α6β4 and α6β1 integrin receptors were shown to associate with the erbB2 receptor in three separate human breast cancer cell lines (Falcioni et al., 1997; Han et al., 1999). In addition, co-operative signaling between the α6β4 and erbB2 receptors were shown to be required for the invasive properties induced by PI3-K (Gambaletta et al., 2000).

Perhaps the most compelling evidence, however, was provided in a series of experiments involving the inhibition of β1-integrin binding activity in human breast carcinoma cells (Wang et al., 2002; Weaver et al., 1997). When cultured in a reconstituted matrix of basement membrane components these tumour cells normally grow as disorganized clusters of rapidly dividing cells. Following the inhibition of β1-integrin activity, however, the cells become growth arrested and revert to well organized 3-dimensional acinar arrangements characteristic of differentiated mammary epithelial cells. In addition, these cells were no longer tumourigenic in immunocompromised mice, unlike their parental counterparts. Consistent with the phenomenon of receptor cross talk observed in normal epithelial cells, the inhibition of β1-integrin activity resulted in the attenuation of EGF receptor phosphorylation and signaling in these human breast cancer cells (Wang et al., 2002; Weaver et al., 1997).

Figure 1.7 Integrins and growth factor receptors engage in intermolecular cross talk

Cell adhesion is required for mediating the biological effects of growth factor receptor signaling. This phenomenon has been explained by the observation that integrin adhesion receptors undergo molecular cross talk with their growth factor receptor counterparts. The collaboration between integrins and growth factor receptors is required for the optimal induction of specific cell signaling pathways, such as those involved in cell cycle progression (as illustrated in the figure). Co-operation between the receptors occurs through the concerted activation of downstream signaling molecules or transcription factors (solid arrows), or through the reciprocal regulation of the receptors themselves (dashed arrows), as described in the text.


BM
β
α
S
S
outside
inside
c-Src
FAK
Grb2
p130CAS
P
P
P
P
Grb2
Ras
SOS
P
P
JNK
P
P
Erk
cyclin D1

Interestingly, a recent report by Liu et al. (2002) showed that the activation of ERK by the EGF receptor in human hepatocarcinoma cells requires a functional complex between β1-integrin and the urokinase plasminogen activator receptor (uPAR), a cell surface receptor associated with tissue remodelling and the growth and dissemination of human cancers (Blasi and Carmeliet, 2002; Liu et al., 2002). Disruption of this complex results in attenuation of FAK-mediated ERK activation, accompanied by the induction of tumour cell dormancy *in vivo*. The ability to inhibit cancer cell proliferation in a variety of cell types by blocking β1-integrin signaling therefore suggests that the β1-integrin subunit may play a universal role in promoting the growth of cells transformed by constitutively active growth factor receptors (Wang et al., 2002).

1.9 Addressing the role of the β1-integrin subunit in mammary gland tumourigenesis *in vivo*

The experimental observations described in the previous sections suggest that the expression and engagement of integrin receptors may be necessary for growth factor receptor-induced cell cycle progression and cell survival in both normal and transformed mammary epithelial cells. The results of these experiments may explain the observation that α6β1 and α6β4 integrin heterodimers have been found to be consistently expressed in human breast cancer tissue (Mercurio et al., 2001; Shaw, 1999). Although tumour-promoting roles for both the β1- and the β4-integin subunits are suggested by experimental and clinical analysis of human breast cancer cells and tissue, the role of the β1 subunit is particularly intriguing, given the striking results of Weaver et al. (1997) and Wang et al. (2002). By using inhibitory anti-β1-integrin antibodies to revert the transformed phenotype of various human breast cancer cell lines, they provide compelling evidence that β1-integrin may be necessary for induction or growth of mammary tumours *in vivo*. This hypothesis, however, has been difficult to test using traditional gene targeting strategies in murine tumour models, since germline deletion of

the β1-integrin gene is embryonically lethal in the homozygous state (Fassler and Meyer, 1995). The recent introduction of tissue-specific gene targeting strategies, however, circumvents this limitation. Chapter 3 in the 'Results' section of this thesis describes the use of a mammary-specific gene targeting approach to determine if β1-integrin expression is required for oncogene-induced mammary tumourigenesis in a mouse model of human breast cancer.

1.10 Effectors of β1-integrin signaling: a potential tumourigenic role for ILK

Experiments involving the manipulation of β1-integrin expression in cultured mammary tumour cells or mouse tumour models are often designed to determine whether the β1-integrin molecule represents a potential target for therapeutic intervention of human breast cancer. Valuable lessons from research on growth factor receptors and human cancer, however, have forced researchers to take into consideration the role of associated intracellular signaling molecules during tumourigenesis and tumour progression. Overexpression or activation of cytoplasmic effectors such as Ras, for example, have been found to be associated with a large proportion of human carcinomas (Hanahan and Weinberg, 2000). In addition, transgenic mouse models overexpressing wild-type or activated forms of these intracellular signaling molecules have provided convincing demonstrations of their potent transforming potential, independent of their activation by growth factor receptors (Hutchinson and Muller, 2000). For therapeutic purposes, therefore, it may be more effective to target these intracellular signaling molecules rather than the upstream growth factor receptors, at least for some tumour types.

In the case of integrin signaling, there is evidence that some of the associated effector molecules are directly involved in the etiology and dissemination of human cancers. The upregulation of FAK, for example, has been found to be correlated with aggressive malignant disease (Cance et al., 2000; Gabarra-Niecko et al., 2003; Oktay et al., 2003; Owens et al., 1995), while experiments designed to inhibit FAK expression and

activity have revealed tumour-promoting roles for this protein both *in vitro* and *in vivo* (Aguirre Ghiso, 2002; McLean et al., 2001). The contribution of FAK to the tumourigenic state is likely due to the transmission of integrin-mediated signals promoting cell cycle progression, survival and migration, which are necessary for tumour progression (Gabarra-Niecko et al., 2003).

In addition to FAK, the integrin-associated ILK has recently attracted a great deal of attention as a potential oncogenic protein (Persad and Dedhar, 2003). ILK is a 59kDa serine/threonine protein kinase, which was cloned by virtue of its association with the cytoplasmic domain of β1-integrin (Hannigan et al., 1996). Not only has ILK been shown to be an important effector of β1-integrin signaling in cultured epithelial cells, but the physical association between ILK and β1-integrin is reflected in a remarkable interdependency *in vivo,* not reported for other β1-integrin-associated molecules. Specifically, loss of the ILK locus in both drosophila and *C.elegans* results in a phenocopy of null-mutations in the β1-integrin gene, including defects in integrin-mediated muscle attachment events (Mackinnon et al., 2002; Zervas et al., 2001). Similarly, targeted germline deletion of either of these genes in mice results in early embryonic lethality in the homozygous state, involving defects in inner cell mass formation and implantation (Fassler and Meyer, 1995; Sakai et al., 2003).

The binding of ILK to the β1-integrin subunit has been mapped to a region in the carboxy terminus of the ILK molecule, which also contains the consensus sequence for the serine/threonine kinase domain (Wu, 2001). Through a series of 4 tandem anykyrin-like repeats in the amino terminus, ILK can also bind to scaffolding proteins such as paxillin, as well as the adaptor protein PINCH, which consists of 4 tandem protein-protein interaction (LIM) domains (Tu et al., 1999; Wu, 2001). Simultaneous binding of ILK to β1-integrin and paxillin or PINCH confers upon ILK an important role in the formation of signaling complexes at cell adhesion sites (Li et al., 1999; Tu et al., 1999). The binding of ILK to PINCH results in a nearly direct association with growth factor receptors such as the PDGF receptor, mediated by the binding of PINCH to the adaptor protein Nck2 (Tu et al., 1999). Through these associations, ILK plays an important role

in facilitating the lateral signaling between integrins and growth factor receptors. Indeed, ILK has been shown to be a point of intermolecular cross talk between β1-integrin and receptors such as the insulin receptor (Delcommenne et al., 1998). In this case, activation of PI3-K by either integrin engagement or insulin results in the induction of ILK kinase activity. The activation of ILK by PI3-K is mediated by the binding of the phosphorylated phosphoinositide lipid products of PI3-K to a pleckstrin homology (PH) domain in the central region of the ILK protein. This interaction then results in the induction of ILK kinase activity and the subsequent phosphorylation of the PKB/Akt anti-apoptotic protein (Delcommenne et al., 1998). Activation of PKB/Akt by ILK therefore provides a molecular mechanism for the activation of an important cell survival pathway by both insulin and integrin-mediated adhesion.

A potential tumourigenic role for ILK was first demonstrated by the overexpression of ILK in cultured epithelial cells (Hannigan et al., 1996). The initial observation involved the morphological transformation of the cells, including the loss of the well-organized 'cobblestone' arrangement characteristic of epithelial cultures . This transition was later found to involve downregulation of the cell-cell adhesion molecule E-cadherin, resulting in the loss of the intercellular epithelial junctions (Wu et al., 1998). This phenomenon was subsequently recapitulated in primary mammary epithelial cells, where the overexpression of ILK induced a transition from an epithelial to a fibroblastic phenotype expressing markers of mesenchymal cells (Somasiri et al., 2001). This epithelial-to-mesenchymal transition (EMT) is often reported in advanced human cancers (Thiery, 2002), therefore providing the first direct indication that ILK has the potential to contribute to an aggressive tumour phenotype. Indeed, the immunohistological analysis of human cancer specimens has subsequently revealed a positive correlation between ILK overexpression and an aggressive malignant phenotype (Ahmed et al., 2003; Dai et al., 2003; Persad and Dedhar, 2003).

Importantly, epithelial cells overexpressing ILK were shown to be tumourigenic in immunocompromised mice (Wu et al., 1998). Molecular and biochemical analysis of these cells revealed the activation of signaling pathways involved in cell cycle

progression and proliferation, in addition to the survival pathway mediated by PKB/Akt. The induction of cell cycle progression and proliferation in these ILK-overexpressing cells resulted from the activation of MAPK and the transcription factor AP-1, as well as transcriptional upregulation of the cyclin D1 gene (Troussard et al., 1999). As described in an earlier section, the activation and expression of these molecules is involved in the integrin-mediated induction of cell cycle progression and survival, suggesting that ILK, in addition to FAK, may be an important effector of these processes. In addition to the activation of these pathways, the overexpression of ILK in cultured epithelial cells results in the stabilization of β-catenin and activation of the β-catenin/LEF-1 transcription complex (Novak et al., 1998). The stabilization of β-catenin by ILK is of great interest with regards to human cancer, since this is known to be an event associated with the induction of colon carcinomas (Bienz and Clevers, 2000).

The overexpression of ILK in cultured epithelial cells, therefore, results in morphological and proliferative alterations characteristic of oncogenic transformation, including tumourigenicity in immunocompromised mice. Human cancers, however, arise following the induction of proliferative pathways *in vivo*. The injection of cells transformed in culture, therefore, does not properly address the oncogenic role of a protein such as ILK. As a result, the second part of the Results section of this thesis presents an experiment designed to test the oncogenic potential of ILK directly *in vivo*, through the establishment of transgenic mice overexpressing ILK in the mammary gland epithelium.

1.11 Summary: Determining the roles of β1-integrin and ILK in mouse mammary gland tumourigenesis

The material reviewed in Chapter 1 was chosen to provide a background to the experiments presented in Chapters 3 and 4 of the Results section of this thesis. Overall, these experiments were designed to answer two important questions regarding the role of β1-integrin receptor signaling during the initiation and progression of mammary gland

carcinoma. The first of these questions is addressed in Chapter 3, where a tissue-specific gene targeting approach is used to determine whether β1-integrin expression is required for mammary tumourigenesis in a mouse model of human breast cancer. As discussed in Chapter 3, the results of this experiment clearly demonstrate that β1-integrin expression, while dispensable for normal mammary gland development, is necessary for the oncogene-induced transformation of the mouse mammary gland epithelium. In addition, the proliferative properties of tumour cells cultured from late-stage (ie. end-point) tumours are shown in these experiments to be dependent upon sustained expression of the β1-integrin subunit. Together, these results provide a clear demonstration that β1-integrin expression is necessary for the initiation and progression of mammary tumourigenesis in this particular mouse model.

Experiments in Chapter 4 were designed to address the second question regarding the role of β1-integrin signaling in mammary tumourigenesis. These experiments involved the use of a transgenic approach, in order to determine whether overexpression of the β1-integrin-associated ILK in the mammary gland epithelium can result in oncogenic transformation. Indeed, transgenic mice overexpressing ILK under the transcriptional control of a mammary-specific promoter developed a hyperplastic mammary gland phenotype by the age of six months, which progressed to the formation of solid mammary gland tumours in a proportion of the animals after one year of age. Evidence of epithelial-to-mesenchymal transition (EMT) in these tumours indicates that the ILK-overexpressing mice may represent a new model of advanced malignant disease.

Together, the results presented in Chapters 3 and 4 of this thesis demonstrate the importance of β1-integrin signaling in mammary tumourigenesis. Importantly, these experiments were performed in a physiologically relevant context, involving the manipulation of these molecules in the mouse mammary gland epithelium *in vivo*. Since the expression of both β1-integrin and ILK are known to be associated with advanced stages of human cancers (Oktay et al., 2003; Persad and Dedhar, 2003), the results presented in this thesis may have clinical and experimental value to the development of therapeutic strategies against human breast cancer.

CHAPTER 2

Materials and Methods

2.1 Animals

MMTV/PyV MT, LoxP1-flanked β1-integrin, MMTV/Cre and GTRosa26 mice were generated and characterized as described elsewhere (Andrechek et al., 2000; Graus-Porta et al., 2001; Guy et al., 1992a; Soriano, 1999). For generation of the MMTV/ILK mice, the 1.8 kb full length cDNA for human ILK (Hannigan et al., 1996) was subcloned into the EcoRI site of plasmid p206, harbouring the MMTV-LTR and the polyadenylation sequence of the SV40 early region (Sinn et al., 1987). The expression cassette was then prepared and injected into one cell zygotes of FVB/N mice, as described previously (Webster et al., 1998). To identify transgenic animals, genomic DNA was isolated from 0.5cm clippings of mouse tails by incubation in DNA lysis buffer (10mM Tris pH 8, 100mM NaCl, 10mM EDTA, 0.5% SDS, 0.2mg/ml proteinase K), overnight at 55°C. Ethanol-precipitated DNA was then PCR amplified using an ILK-specific forward primer (5'-CATGTATGCACCTGCCTG) and an SV40-specific reverse primer (5'-TATGTCACACCACAGAAG), to generate a transgene-specific amplification product. PCR conditions included a 30 second annealing step at 52 °C, and a 1 minute extension at 72°C, for 30 cycles.

All animals were housed in the Central Animal Facility at McMaster University, under compliance with the Animal Research Ethics Board (AREB). To preclude the potential confounding issue of genetic background effects, the $\beta 1^{LoxP1/LoxP1}$ mice used in Chapter 3 were backcrossed onto the FVB genetic background, identical to that of the MMTV/Cre and MMTV/PyV MT strains used in these experiments.

2.2 RNA isolation

Tissue RNA was isolated by the method of CsCl sedimentation (Chirgwin et al., 1979). The tissue was first flash frozen in liquid nitrogen and homogenized in 3ml of a guanidine isothiocyanate (GIT) solution (4M GIT, 25mM sodium citrate, 0.1M β-mercaptoethanol). The tissue homogenate was then layered onto 4mls of 5.7M CsCl containing 25mM sodium acetate (pH 5.2), followed by ultracentrifugation at 32,000 rpm (SW41Ti rotor) for 18 hours at 20°C. The RNA pellet was resuspended in 500μl of sterile, DEPC-treated water, extracted with phenol:chloroform (1:1), and precipitated with 2 volumes of ethanol.

2.3 RNase protection analysis for transgene expression

RNase protection analysis was performed according to the protocol of Melton et al. (1984) (Melton et al., 1984), using a riboprobe specific for the polyadenylation sequence of the SV40 component of the MMTV-driven transgene (Webster et al., 1998). RNA (15-30μg) was incubated with the transgene-specific riboprobe in hybridization buffer (80% formamide, 40mM PIPES, 1mM EDTA, 400mM NaCl) for 5 mins at 85°C. RNA and riboprobe were then allowed to hybridize overnight at 50°C. Non-hybridized regions were digested in RNase digestion buffer (300mM NaCl, 10mM Tris pH 7.4, 5mM EDTA, 2μg/ml RNase T1, 40μg/ml RNase A) for 20 mins at 37°C. Reactions were terminated by adding 500μl phenol:chloroform (1:1), and the protected RNA fragments were precipitated in ethanol, after the addition of 30μg tRNA. For electrophoresis, RNA was resuspended in 10μl of loading buffer (80% formamide, 10mM EDTA, 1mg/ml xylene cyanol, 1mg/ml bromophenol blue), and boiled for 5 mins. Protected fragments were then resolved on a 6% polyacrylamide gel containing urea, dried and exposed overnight at -70°C.

2.4 Protein extractions and immunoblot analysis

Mammary gland and tumour samples were flash frozen in liquid nitrogen, and lysed in buffer containing 50mM HEPES, pH 7.5, 150mM NaCl, 10% glycerol, 1% triton X-100, 1mM EGTA, 2mM EDTA, 10mM NaF, 10mM Napyrophosphate, 1ug/ul leupeptin, 1 ug/ul aprotinin and 1mM Na orthovanadate. Protein concentrations were determined using the Bio-Rad protein assay kit. Samples (20-40μg) were then electrophoresed through a 12% PAG, and transferred to an immobilin-P nylon membrane. Membranes were blocked in 3% nonfat dried milk in 1xTBS, 0.05% Tween-20, incubated in primary antibody overnight at 4°C, washed in TBS/0.05% Tween-20, and incubated with HRP-conjugated secondary antibody (Jackson Immunoresearch Laboratories) for 1 hour at room temperature. Secondary antibody was visualized using ECL reagent, according to the manufacturer's instructions. In the case of phospho-protein analysis, blots were stripped in 2%SDS/β-mercaptoethanol/Tris (pH 6.8) at 70°C for 30 minutes, blocked, and reprobed with antibodies recognizing total (phosphorylated and unphosphorylated) protein.

2.5 Antibodies

Primary antibodies used in these experiments include anti-β1-integrin rabbit polyclonal (M-106) (Santa Cruz), anti-ILK rabbit polyclonal (Upstate), anti-cytokeratin 8 (NCL-CK8-TS1) (Novocastra), anti-phospho-PKB/Akt, anti-phospho-GSK-3β and anti-phospho-MAPK polyclonal antibodies (Cell Signaling Technology), anti-Cre monoclonal (MMS-106A) and rabbit polyclonal (PRB-106C) (Covance), anti-Ki67 monoclonal (MM1) (Novocastra), anti-cyclin D1 monoclonal (72-13G) (Santa Cruz), anti-PyV MT monoclonal (Pab 762) (gift of Dr. Steven Dilworth), anti-FAK, anti-pFAK (Y576), anti-pFAK (Y397) rabbit polyclonals (Upstate), anti-c-Src monoclonal (GD11) (Upstate), and anti-pC-Src (PyV416) polyclonal (Cell Signaling Technology).

2.6 Analysis of Cre-mediated recombination and Cre expression

For PCR analysis of Cre-mediated recombination of the LoxP1-flanked β1-integrin allele, DNA was extracted from mammary gland and tumour tissue as described in section 2.1. The DNA was then PCR amplified, using primers (5'-GCCGCCACAGCTTTCTGCTGTAGG; 5'-GGCCCTGCTTGTATACATTCTCCGC) common to all three forms of the β1-integrin gene (wild-type (1.8kb), LoxP1-flanked (2.1kb), and recombined (1.3kb)). As indicated, the amplification of these alleles produces discrete products of different molecular weight, which can be easily distinguished by agarose gel electrophoresis (Graus-Porta et al., 2001). The PCR reaction conditions were as follows: 94°C for 5min (step 1); 94°C for 45sec (step 2); 60°C for 45sec (step 3); 72°C for 3min (step 4); repeat steps 2-4 for 29 cycles, and finish with 72°C for 5mins.

For RT-PCR analysis of Cre expression, 50ng of total RNA was reverse transcribed using an oligo-dT primer. The RNA was isolated and purified as described in section 2.2, and the first strand reaction was carried out according to the protocol provided with Superscript II® Reverse Transcriptase (GIBCO). PCR amplification was then performed on 2μl of the first strand reaction product, using primers specific for the bacteriophage P1 Cre recombinase (5'-TGCTCTGTCCGTTTGCCG; 5'-ACTGTGTCCAGACCAGGC).

2.7 Immunofluorescence and immunohistochemistry of tissue sections

Tissue samples were either flash frozen directly in Tissue-Tek® OCT compound (Sakura) in a liquid nitrogen bath, or fixed overnight in formalin and embedded in paraffin blocks. Sections were prepared at 5μm thickness. Prior to staining, paraffin-embeded samples were de-paraffinized in 3 changes of xylenes, then hydrated by passing through alcohol (100%, 70% ethanol) to PBS. Antigen retrieval was performed by microwaving the samples in 10mM sodium citrate (pH 6). Endogenous tissue peroxidase activity was then inhibited by incubation in 3% H_2O_2/PBS for 20 minutes. Sections were

incubated in primary antibody (1:100) for 1 hour at room temperature. After washing in PBS, sections were then incubated in FITC- or Cy3-labelled (IF) (Molecular Probes) or HRP-conjugated (IHC) (Jackson Immunoresearch Laboratories) secondary antibody (1:1000) for 30 minutes at RT.

2.8 Immunofluorescence microscopy of cultured cells

Cells were plated in treated glass slide chambers, and fixed with 2% paraformaldehyde for 20 minutes. Cells were then permeabilized with 0.5% Triton X-100 in PBS, for 10 minutes at room temperature. Primary antibodies were diluted in blocking buffer (0.2% Triton X-100; 0.05% Tween-20; 3% BSA in PBS) at a dilution of 1:100, and applied to cells for 1 hour at RT. After washing 3 times in PBS, cells were incubated with FITC- or Cy3-labelled secondary antibodies (Molecular Probes) diluted 1:1000 in blocking buffer, for 30 minutes at room temperature. Cells were counterstained with DAPI, and photographed on a Zeiss 510 confocal microscope.

2.9 Mammary gland wholemounts

Number 3 or 4 mammary glands were spread on glass slides, and defatted overnight in acetone. The glands were then stained in Harris' hematoxylin (Fisher Scientific) for 3 hours, followed by destaining in 1%HCl/70% ethanol, to remove non-specific background stain. The glands were dehydrated through 100% ethanol and xylenes, then mounted under glass coverslips using Permount® (Fisher Scientific).

2.10 *In situ* β-galactosidase assay

Glands were spread on 35mm cell culture dishes and fixed for 1 hour in 2% paraformaldehyde containing 0.25% glutaraldehyde and 0.01% NP-40. The glands were then rinsed in PBS and incubated in the staining solution (1xPBS; 2mM MgCl2; 0.01% sodium deoxycholate; 0.02% NP-40; 1mg/ml 5-bromo-4-chloro-3-indolyl-B-D-

galactoside (Xgal) (Invitrogen)) overnight at room temperature. Stained glands were then passed through ethanol (70%, 100%) and xylenes, prior to mounting. For sectioning, tumours and glands were stained as above then embeded in paraffin. Slides were counterstained with Eosin Yellowish solution (Fisher Scientific) for clear delineation of β-galactosidase-expressing cells.

2.11 Tumor cell explants and adenovirus infection

Pieces of tumor (200mg) were removed from the mammary glands of female MMTV/PyV MT mice, harboring either the $\beta1^{LoxP1/LoxP1}$ or $\beta1^{LoxP1/wt}$ genotype. Samples were rinsed in sterile PBS and transferred to a clean 10cm cell culture dish, where they were minced to homogeneity using a flat razor blade. The tumor tissue was then added to a solution of DMEM containing 2mg/ml collagenase B (Roche), and incubated for 1 hour at 37°C, with constant agitation. Cells were then washed 3 times in DMEM, including centrifugation at 800rpm to selectively bring down epithelial cell aggregates, and plated in 10% FBS/DMEM. Cells were infected with adenovirus vector (gift of Dr. Frank Graham) at 50moi for 30 minutes in DMEM, washed, and refed with complete medium. Analysis for β-galactosidase expression, Cre-mediated excision of LoxP1-flanked β1-integrin alleles, and protein levels were determined at 4 days post-infection.

2.12 Tumor cell transplants

All surgeries were performed in accordance with AREB guidelines. The number 4 glands of anaesthatized, 3 week-old female FVB mice were surgically exposed under sterile conditions. Cells suspended in 10ul sterile PBS were then injected into the fat pad, using a Hamilton syringe, at a site proximal to the abdominal wall and distal to the nipple. The entire first quadrant of the fat pad, containing the lymph node and nipple, was then removed by cauterization. Experimental and control cells were injected contralaterally, and the mice were sutured and allowed to recover under supervision.

CHAPTER 3

β1-integrin expression is required for induction and maintenance of the mammary tumour phenotype in a mouse model of human breast cancer

3.1 INTRODUCTION

The β1-integrin subunit is expressed in clinical samples of human breast cancer (Mercurio et al., 2001; Shaw, 1999). Moreover, expression of the β1-integrin-containing α6β1-integrin receptor has been shown to be correlated with an aggressive, high grade tumour phenotype, while overexpression of the α6 binding partner is associated with low survival rates in breast cancer patients (Friedrichs et al., 1995; Shaw, 1999). These expression patterns in clinical tumour samples therefore suggest a potentially important role for integrin receptors containing the β1 subunit in the etiology and progression of human breast cancer. A potential tumour-promoting role for β1-integrin is supported by experiments involving manipulation of the β1-integrin subunit in human breast cancer cell lines. In one particularly important set of experiments, the tumorigenic phenotype of the tumour cells was reversed by blocking the binding properties of the β1-integrin subunit, using an inhibitory anti-β1-integrin antibody (Wang et al., 2002; Weaver et al., 1997). After culturing the cells in the presence of reconstituted basement membrane components and the inhibitory anti-β1-integrin antibody, the large, irregular clusters characteristic of transformed cells were converted to well-differentiated acini, accompanied by the restoration of normal cell-cell junctions. Importantly, these cells were no longer tumourigenic in nude mice following their phenotypic reversion in the cell culture environment.

While these observations suggest that β1-integrin is required for induction and/or maintenance of the tumour phenotype in human breast cancer cells, the physiological significance of β1-integrin expression during tumourigenesis has been difficult to ascertain *in vivo*. This limitation is primarily due to the embryonic lethality of the

targeted deletion in the β1-integrin gene (Fassler and Meyer, 1995), as discussed in Chapter 1. As a result, a conditional allele of β1-integrin (Graus-Porta et al., 2001) was introduced into a mouse model of human breast cancer in order to target the ablation of β1-integrin expression to the post-natal mammary gland epithelium. This strategy was designed to determine if β1-integrin expression is required for mammary tumourigenesis in a physiologically relevant context.

The tumour model used in these experiments expresses the polyomavirus (PyV) middle-T (MT) antigen under the transcriptional control of the mouse mammary tumour virus (MMTV) long terminal repeat (LTR), which has promoter/enhancer activity specifically in the mouse mammary gland epithelium (Guy et al., 1992a). Female mice expressing the MMTV/PyV MT transgene develop metastatic, multifocal mammary gland tumours, palpable by the age of 2 to 3 months (Guy et al., 1992a). The potent transforming properties of the PyV MT antigen have been attributed to the constitutive activation of pathways involved in proliferation and survival (Ichaso and Dilworth, 2001). The activation of these pathways is through a direct association with several important cell signaling molecules, including c-Src, PI3-K and Shc (Figure 3.1.1). The expression of PyV MT mutants lacking binding sites for these molecules has demonstrated the importance of each molecule during aspects of mammary tumourigenesis and metastasis in transgenic mice (Webster et al., 1998). The activation of c-Src, in particular, seems to be critical for the transforming properties of the PyV MT antigen in the mouse mammary gland (Guy et al., 1994).

The constitutive activation of c-Src is believed to occur through the binding of the PyV MT molecule to an inhibitory residue in the carboxy terminus of c-Src (tyrosine 527) (Figure 3.1.1) (Ichaso and Dilworth, 2001). The occupation of this site by the amino terminus of PyV MT presumably facilitates an open conformation of the c-Src molecule, exposing the ATP-binding pocket and the catalytic domain (Ichaso and Dilworth, 2001; Xu et al., 1999a; Xu et al., 1997). In this active conformation, the c-Src kinase then constitutively phosphorylates tyrosine residues on immediate downstream targets such as FAK (Figure 3.1.2) (Ichaso and Dilworth, 2001). The presence of

Figure 3.1.1 The polyomavirus (PyV) middle-T (MT) antigen induces cell proliferation and transformation through the binding of intracellular signaling molecules

By recruiting various cell signaling molecules to the inner side of the membrane, the PyV MT protein induces activation of cell signaling pathways involved in cell proliferation and survival. Constitutive activation of the c-Src kinase, in particular, has been shown to be critical for the transforming properties of the PyV MT molecule. The kinase activity of c-Src is induced by exposure of an ATP binding site in the activation domain (Y416), following association of the PyV MT protein with the inhibitory c-Src tyrosine residue 527 (Y527). Since the PyV MT-associated molecules are also recruited by phosphorylated receptor tyrosine kinases, the PyV MT antigen is considered a constitutively activated analogue of growth factor receptors.

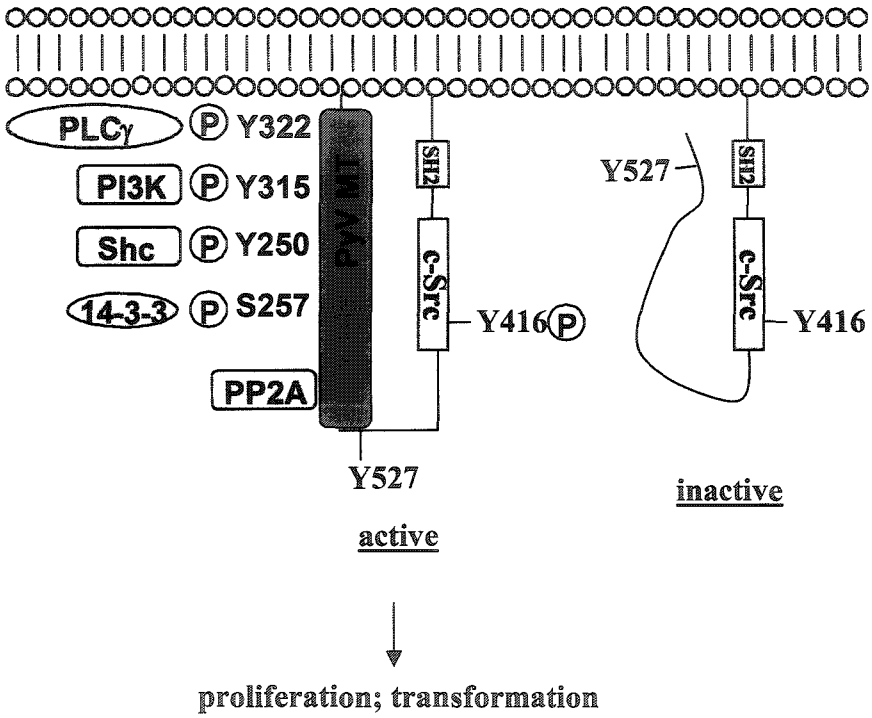
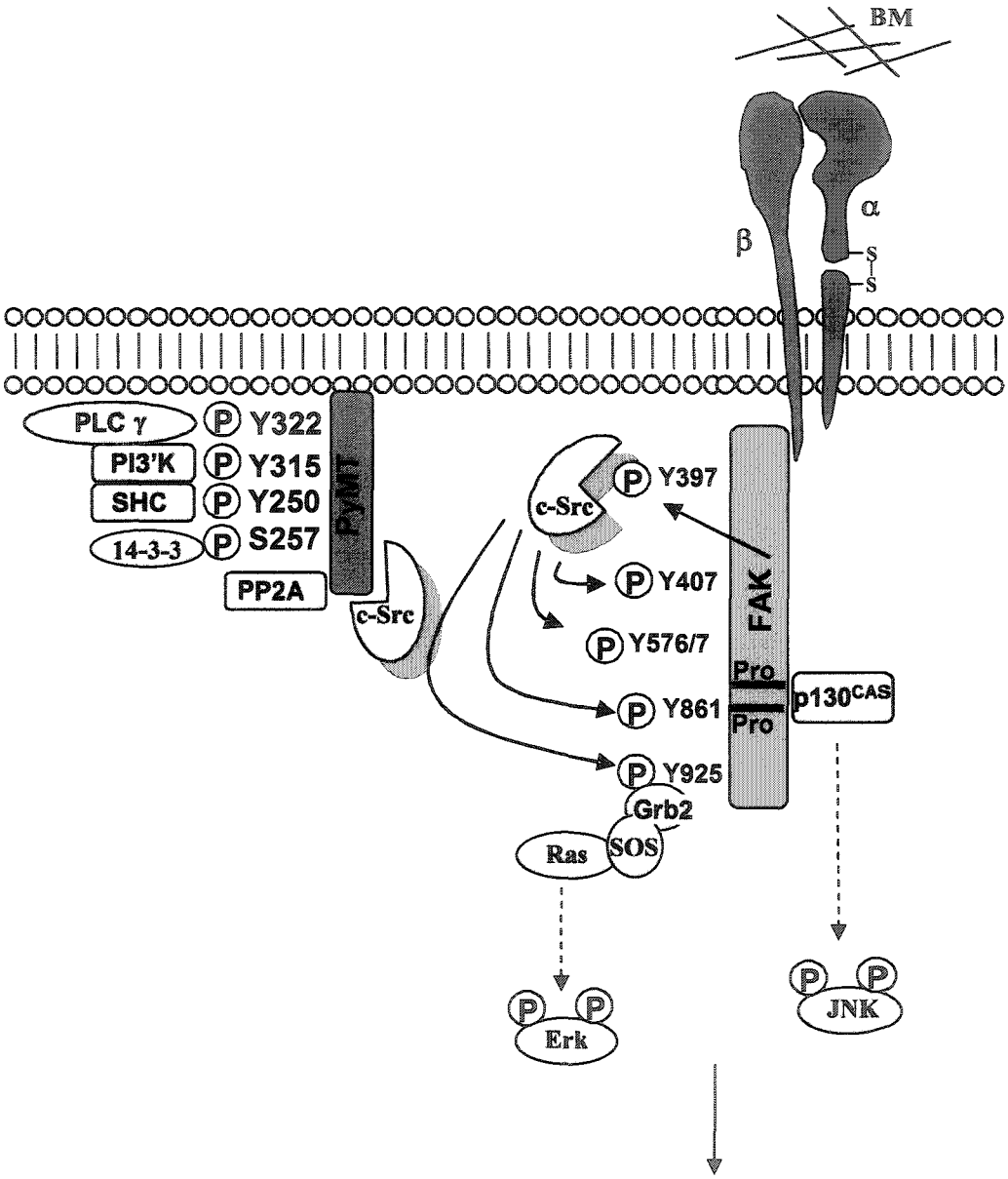

PLCγ
P Y322
PI3K
P Y315
Shc
P Y250
14-3-3
P S257
PP2A
PY NT
SH2
c-Src
Y416P
Y527
active
Y527
SH2
c-Src
Y416
inactive
proliferation; transformation

Figure 3.1.2 FAK mediates the biological properties of integrin receptors and the c-Src kinase

FAK is recruited to the inner cell membrane at sites of integrin clustering, where autophosphorylation facilitates binding of the c-Src kinase at tyrosine 397 (Y397). Following c-Src-mediated phosphorylation of additional tyrosine residues along the FAK molecule, FAK recruits and activates several intracellular effectors of cell proliferation, migration and survival. In this way FAK occupies a central position to mediate the signaling properties of both the integrin receptors and the c-Src kinase. A role for FAK in mediating the transforming function of PyV MT-associated c-Src is shown in the model.



proliferation; migration; survival

phosphorylated tyrosine residues along the carboxy terminus of the FAK protein subsequently provides binding sites for additional signaling molecules such as Grb2, which are important for mediating the cell signaling properties of the FAK molecule. The phosphorylation of FAK by c-Src has been shown to be important for mediating the biological properties of both c-Src and β1-integrin signaling, including the induction of cell cycle progression and the regulation of focal adhesion turnover during cell migration (Oktay et al., 1999; Webb et al., 2004; Zhao et al., 1998). In addition, FAK has been shown to play a critical role during the induction of oncogenic transformation by constitutively activated versions of c-Src (Xing et al., 1994).

The short latency of tumour induction, combined with 100% penetrance of the tumour phenotype (Guy et al., 1992a), confer upon the MMTV/PyV MT mice a degree of convenience not exhibited by other tumour models. These phenotypic aspects of these mice make them particularly attractive for studies involving multiple genetic crosses, such as those described below. More importantly, however, extensive molecular and pathological analysis of the MMTV/PyV MT model has revealed that these mice provide a reliable recapitulation of many aspects of human breast cancer (Lin et al., 2003; Maglione et al., 2001). Tumours arising in MMTV/PyV MT mice pass through several pathological stages reported in human malignancies, including the early atypical hyperplasias, which are accompanied by identical changes in the expression of molecular markers such as cyclin D1 (Lin et al., 2003). The reliability of this model is likely due to the PyV MT-induced activation of downstream signaling molecules including c-Src, PI3-K and MAPK, which are known to be activated by oncogenic growth factor receptors such as erbB2. Indeed, signaling from the PyV MT protein has been described as a surrogate for erbB2 receptor signaling (Cardiff et al., 2000). The MMTV/PyV MT mice therefore provide a physiologically relevant model to determine if β1-integrin expression is required for mammary tumourigenesis *in vivo*.

3.2 RESULTS

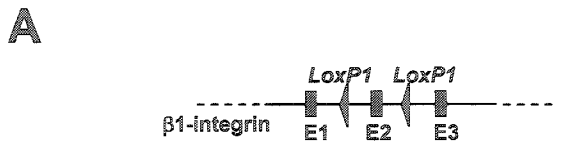
3.2.1 A conditional β1-integrin allele can be efficiently excised from the murine mammary gland epithelium

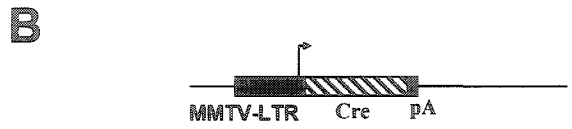
The approach used in these experiments involves the use of Cre/LoxP1 technology in order to excise a conditional allele of β1-integrin (Figure 3.2.1A) (Graus-Porta et al., 2001) in the mammary glands of the MMTV/PyV MT mammary tumour model. This technology is based on the enzymatic activity of the bacteriophage P1 Cre recombinase (Cre), which induces homologous recombination between short (34bp) nucleotide sequences (LoxP1 sites). If the LoxP1 sites are placed on the flanking ends of a gene or cDNA, then the intervening sequence will be excised following Cre-induced recombination (Sauer, 1998). As a result, tissue-specific excision of a LoxP1-flanked genomic sequence, such as that shown in Figure 3.2.1A, can be induced *in vivo* by the expression of Cre under a tissue-specific promoter (Le and Sauer, 2001).

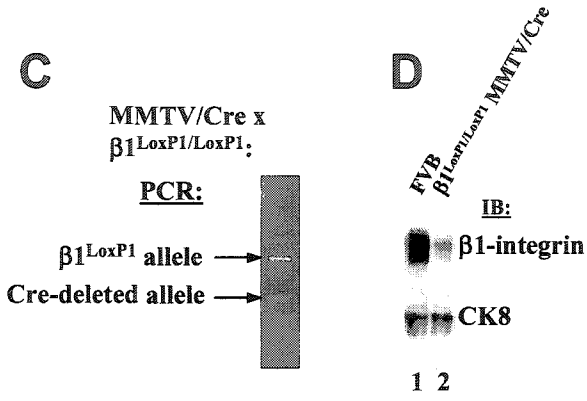
In order to facilitate Cre-mediated excision of the LoxP1-flanked β1-integrin ($\beta1^{LoxP1}$) allele specifically in the mouse mammary gland epithelium, the MMTV-LTR was used to drive Cre expression in a $\beta1^{LoxP1/LoxP1}$ background (the MMTV/Cre construct (Andrechek et al., 2000) is shown in Figure 3.2.1B). This was achieved by successively breeding the MMTV/Cre and $\beta1^{LoxP1}$ strains until the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre genetic combination was obtained. As shown in Figure 3.2.1C, mammary-specific excision of the $\beta1^{LoxP1}$ allele could be confirmed in these $\beta1^{LoxP1/LoxP1}$ MMTV/Cre animals by PCR amplification of mammary gland DNA, using primers which amplify both the intact and Cre-deleted forms of the $\beta1^{LoxP1}$ allele (the Cre-deleted form is the lower band in Figure 3.2.1C—amplification of the intact $\beta1^{LoxP1}$ allele likely reflects the presence of stromal cell DNA in the preparation). To confirm that this excision event was accompanied by a reduction in β1-integrin protein levels, an anti-β1-integrin antibody was used to perform immunoblot analysis on protein lysates prepared from the mammary epithelial cells of

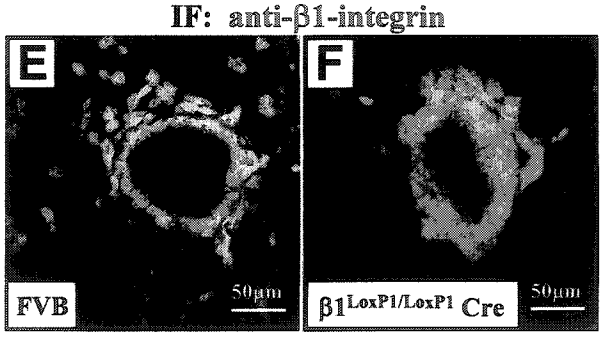
Figure 3.2.1 Excision of a conditional β1-integrin allele can be targeted to the mouse mammary gland epithelium

A conditional allele of β1-integrin was generated by flanking the second coding exon of the β1-integrin locus with LoxP1 recombination sites (**A**). This allele can be excised in the mouse mammary gland following expression of the Cre recombinase under transcriptional control of the mouse mammary tumour virus (MMTV) long terminal repeat (LTR) (**B**). Excision of the $\beta1^{LoxP1}$ allele in the mammary gland of a $\beta1^{LoxP1/LoxP1}$ MMTV/Cre mouse was confirmed by PCR analysis of mammary gland DNA, using primers which amplify the intact and Cre-deleted forms of the $\beta1^{LoxP1}$ allele (**C**). A corresponding reduction in β1-integrin protein levels was confirmed by immunoblot analysis of mammary epithelial cell protein lysates (**D**), and by immunofluorescence analysis of FVB (**E**) and $\beta1^{LoxP1/LoxP1}$ MMTV/Cre (**F**) derived mammary gland cross sections. Both analyses were performed using an anti-β1-integrin polyclonal antibody. Red signal from a Cy3-labeled secondary antibody indicates β1-integrin protein expression in panel E.


A
LoxP1
LoxP1
β1-integrin
E1
E2
E3


B
MMTV-LTR
Cre
pA


C
MMTV/Cre x
β1LoxP1/LoxP1:
PCR:
β1LoxP1 allele
Cre-deleted allele
D
FVB
β1LoxP1/LoxP1 MMTV/Cre
IB:
β1-integrin
CK8
1 2


IF: anti-β1-integrin
E
F
FVB
50μm
β1LoxP1/LoxP1 Cre
50μm

$\beta1^{LoxP1/LoxP1}$ MMTV/Cre mice (Figure 3.2.1D). Note that in contrast to lysates prepared from FVB control mice (Figure 3.2.1D, lane 1), protein preparations from $\beta1^{LoxP1/LoxP1}$ MMTV/Cre animals contained dramatically lower levels of β1-integrin protein (Figure 3.2.1D, lane 2). Using the same anti-β1-integrin antibody, the specificity of this Cre-mediated depletion of β1-integrin expression could be demonstrated by immunofluorescent analysis of frozen mammary gland sections from $\beta1^{LoxP1/LoxP1}$ MMTV/Cre mice. While β1-integrin protein is normally expressed along the basolateral surface of mammary epithelial cells in control FVB mice (Figure 3.2.1E, red staining), expression of the protein was found to be absent in most of the stained sections from $\beta1^{LoxP1/LoxP1}$ MMTV/Cre animals (Figure 3.2.1F). Introducing 2 copies of the $\beta1^{LoxP1}$ allele into MMTV/Cre mice therefore results in Cre-mediated excision of the $\beta1^{LoxP1}$ sequence, accompanied by a reduction in β1-integrin protein levels in the epithelial compartment.

3.2.2 Mammary-specific ablation of β1-integrin expression does not interfere with the initial stages of mammary ductal outgrowth nor epithelial cell viability

Prior to the introduction of this conditional β1-integrin allele into the MMTV/PyV MT mouse tumour model, however, it was necessary to determine if targeted ablation of β1-integrin was indeed compatible with normal mammary gland development in these animals. Although this targeted, Cre-mediated approach circumvents the embryonic lethality associated with germline ablation of the β1-integrin gene (Fassler and Meyer, 1995), there remains the possibility that excision of the $\beta1^{LoxP1}$ sequences *in vivo* may have undesirable consequences for mammary gland development in the mice. This concern is based on three major lines of evidence. First of all, receptors containing the β1-integrin subunit are expressed throughout the mammary gland epithelium, in both the luminal and myoepithelial cell populations (Taddei et al., 2003). In addition, ligands of β1-integrin, such as laminin-1, are distributed around the epithelial ducts of the developing and adult mammary gland (Prince et al., 2002). The

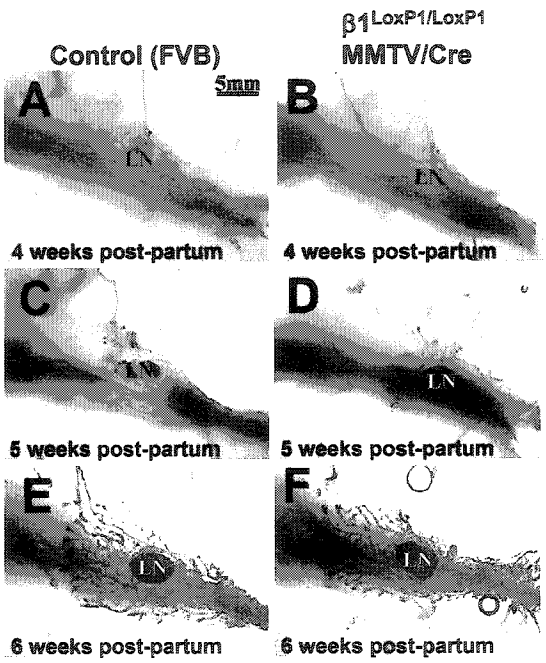
distribution of the β1-integrin subunit and its ligands, therefore, implies that β1-integrin expression may have physiological importance for mammary gland physiology. Second, the presence of BM-derived ligands of β1-integrins have been shown to be required for mammary epithelial cell survival, differentiation and proliferation in cell culture-based studies (Farrelly et al., 1999; Streuli et al., 1995). Third, an experiment designed to inhibit the binding activity of laminin receptors, including β1-integrin, in the developing gland resulted in a reduced number of terminal end bud structures and impaired ductal outgrowth (Klinowska et al., 1999).

As a result, mammary gland wholemounts were prepared from β1 $^{LoxP1/LoxP1}$ MMTV/Cre mice at 4, 5 and 6 weeks of age, encompassing the time of maximum growth of the gland during puberty (Figure 3.2.2.1B, D and F). These glands were then compared to those from FVB control mice (Figure 3.2.2.1A, C and E), to determine if there were any abnormalities in development of the β1 $^{LoxP1/LoxP1}$ MMTV/Cre-derived glands at these time points. However, no differences in either the rate of ductal outgrowth (relative to the lymph node) or the extent of ductal branching could be detected between the 2 sets of glands, indicating that the β1 $^{LoxP1/LoxP1}$ MMTV/Cre genetic combination had no observable impact on normal mammary gland development in this model. The significance of this result, relative to the other observations described above, will be discussed later.

There are unpublished reports that MMTV-LTR promoter activity in the mouse mammary epithelium is stochastic. It is therefore conceivable that the growth of the gland in β1$^{LoxP1/LoxP1}$ MMTV/Cre mice was supported by the preferential proliferation of cells retaining expression of β1-integrin. Testing this hypothesis would require an efficient method to distinguish, *in situ*, between cells undergoing Cre-mediated excision and those not expressing the MMTV/Cre transgene. For this purpose, we decided to introduce a Cre-responsive β-galactosidase reporter construct into the β1$^{LoxP1/LoxP1}$ MMTV/Cre combination, by breeding with the GTRosa26 line of mice (Soriano, 1999). This GTRosa26 reporter gene was constructed in such a way that excision of a transcriptional stop sequence by Cre results in β-galactosidase expression from the

Figure 3.2.2.1 Mammary gland development is not impaired in the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre genetic background

Mammary gland outgrowth in the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre background was compared to that of control FVB mice, at 3 time points spanning the period of maximum growth during puberty. The distance of migration into the fat pad (relative to the position of the lymph node—LN) and the degree of ductal branching revealed that excision of the $\beta1^{LoxP1}$ alleles in the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre background did not impair mammary gland development in these animals.


Control (FVB)
β1LoxP1/LoxP1
MMTV/Cre
5mm
A
B
LN
LN
4 weeks post-partum
4 weeks post-partum
C
D
LN
LN
5 weeks post-partum
5 weeks post-partum
E
F
LN
LN
6 weeks post-partum
6 weeks post-partum

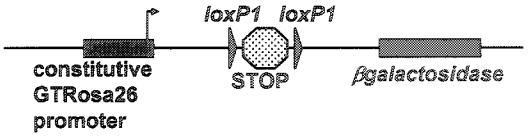
constitutve GTRosa26 mouse promoter/enhancer (Figure 3.2.2.2A). Mammary epithelial cells expressing the MMTV/Cre transgene can therefore be identified *in situ* using a colourimetric, Xgal-based assay for β-galactosidase activity. As shown in Figure 3.2.2.2B, this approach was used to provide the first convincing demonstration of the stochastic nature of MMTV-LTR promoter activity in the mouse mammary gland. Representative sections of stained glands from MMTV/Cre GTRosa26 bitransgenic animals revealed that the MMTV/Cre transgene was expressed in anywhere from 100% to 50% of the luminal epithelial cell population, depending on the particular section (Representative sections are shown in the three panels of Figure 3.2.2.2B, where blue staining indicates Cre activity).

To determine whether there was preferential retention of β1-integrin-positive cells in the developing glands of $\beta1^{LoxP1/LoxP1}$ MMTV/Cre mice, therefore, X-gal-stained mammary gland wholemounts were prepared from 12 week-old MMTV/Cre GTRosa26 animals harbouring either 1 or 2 copies of the $\beta1^{LoxP1}$ allele (Figure 3.2.2.3). When these glands were compared at both low (Figure 3.2.2.3A and B) and high (Figure 3.2.2.3C and D) powers of magnification, no differences could be detected with regards to the proportion of cells undergoing Cre-mediated excision events (indicated by blue staining). Since the only genetic difference between the GTRosa26 $\beta1^{LoxP1/LoxP1}$ MMTV/Cre and GTRosa26 $\beta1^{LoxP1/wt}$ MMTV/Cre control animals was the presence of two $\beta1^{LoxP1}$ alleles in the former, this result suggests that β1-integrin-null cells are not at a selective disadvantage during development of the normal mouse mammary gland. The absence of developmental complications in the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre background therefore indicates that the MMTV/Cre-mediated excision of $\beta1^{LoxP1}$ alleles *in vivo* represents a viable approach for examining the role of β1-integrin expression during mammary tumourigenesis in the MMTV/PyV MT model.

Figure 3.2.2.2 Introduction of a Cre-responsive β-galactosidase reporter reveals stochastic expression of the MMTV/Cre transgene

MMTV/Cre-expressing cells can be identified *in situ* by introduction of the GTRosa26 reporter construct (A), where Cre-mediated excision of a transcriptional stop sequence facilitates expression of a β-galactosidase reporter from the constitutive GTRosa26 mouse promoter/enhancer (Soriano, 1999). (B) Representative sections of a mammary gland from a GTRosa26 MMTV/Cre mouse. The glands were stained with the colourimetric β-galactosidase substrate Xgal prior to sectioning, in order to identify MMTV/Cre-expressing cells (blue). Note the variability in the proportion of MMTV/Cre-expressing cells between these samples.

A


loxP1 loxP1
constitutive
GTRosa26
promoter
STOP
βgalactosidase

B

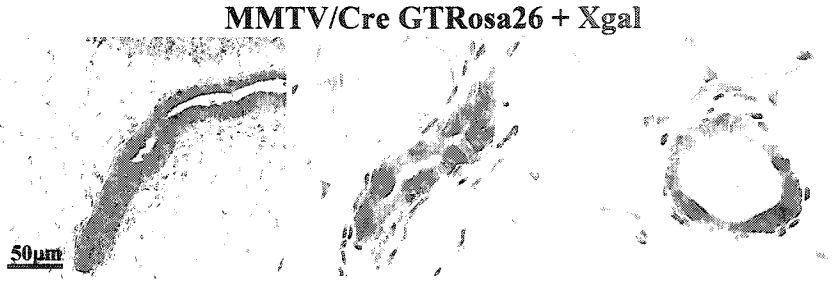
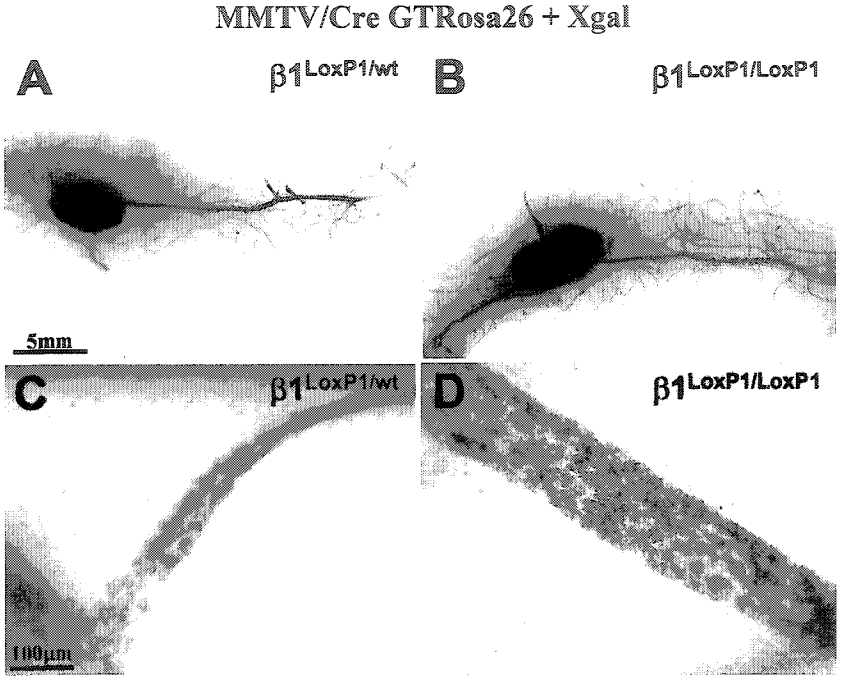

MMTV/Cre GTRosa26 + Xgal
50μm

Figure 3.2.2.3 Cre-expressing cells contribute equally to mammary gland development in the $\beta1^{LoxP1/wt}$ and $\beta1^{LoxP1/LoxP1}$ backgrounds

The GTRosa26 reporter was used to determine if Cre-expressing cells were at a selective disadvantage in the mammary glands of $\beta1^{LoxP1/LoxP1}$ mice. The proportion of Cre-expressing cells (blue) visible in the overall glandular epithelium (**A, B**) and ductal regions (**C, D**) were found to be equivalent between the $\beta1^{LoxP1/wt}$ MMTV/Cre (**A, C**) and $\beta1^{LoxP1/LoxP1}$ MMTV/Cre (**B, D**) backgrounds. Excision of both $\beta1^{LoxP1}$ alleles, therefore, does not confer a selective disadvantage for Cre-expressing $\beta1^{LoxP1/LoxP1}$ epithelial cells in the developing gland.


MMTV/Cre GTRosa26 + Xgal
A
β1LoxP1/wt
B
β1LoxP1/LoxP1
5mm
C
β1LoxP1/wt
D
β1LoxP1/LoxP1
100μm

3.2.3 The number of hyperplastic mammary lesions in MMTV/PyV MT mice is reduced in the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre background.

The distribution of β1-integrin protein in the hyperplastic mammary glands of MMTV/PyV MT mice suggests that there is a correlation between β1-integrin expression and oncogenic transformation in these animals (Figure 3.2.3.1). Specifically, immunohistochemical analysis of MMTV/PyV MT-derived mammary gland sections, using an anti-β1-integrin antibody, revealed that β1-integrin protein was expressed throughout regions of the mammary epithelium undergoing hyperplastic expansion (Figures 3.2.3.1, black asterisks). Moreover, expression of β1-integrin in these hyperplastic regions was found to be markedly higher than in the adjacent, single-layered epithelium (Figures 3.2.3.1, green arrowheads), using this staining protocol. To determine whether this expression pattern reflects a requirement for β1-integrin expression during PyV MT-induced transformation in these animals, the MMTV/PyV MT transgene was introduced into the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre background, as well as a control background heterozygous for the $\beta1^{LoxP1}$ allele ($\beta1^{LoxP1/wt}$ MMTV/Cre). By the age of 10 weeks, mice expressing the MMTV/PyV MT transgene in the control background exhibit multiple neoplastic mammary lesions, occupying approximately 70% to 80% of the total epithelial surface area (Figure 3.2.3.2A). When the MMTV/PyV MT transgene was introduced into the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre background, however, the number of lesions was found to be dramatically reduced, encompassing less than 20% of the gland (Figure 3.2.3.2B). This result was reproducible in 7 out of 7 comparisons between glands of age-matched $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT and control $\beta1^{LoxP1/wt}$ MMTV/Cre/PyV MT mice suggesting that ablation of β1-integrin expression can dramatically impair mammary gland transformation in the MMTV/PyV MT mice.

Figure 3.2.3.1 β1-integrin protein is expressed during PyV MT-induced transformation of the mammary gland

Mammary gland sections from 10 week-old MMTV/PyV MT mice were incubated with an anti-β1-integrin antibody and an HRP-conjugated secondary antibody. Prominent staining for β1-integrin protein (brown) was visible in regions of the mammary epithelium undergoing hyperplastic expansion (asterisks). When compared to the relatively weak staining in single-layered regions of the epithelium (green arrowheads), this results suggests that β1-integrin is upregulated during PyV MT-induced transformation.

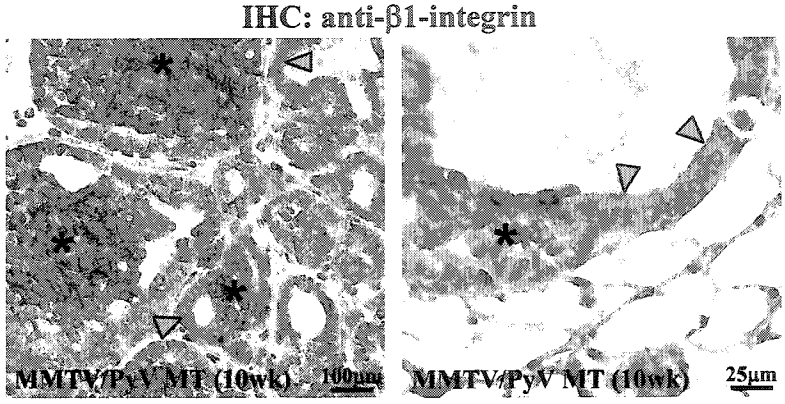
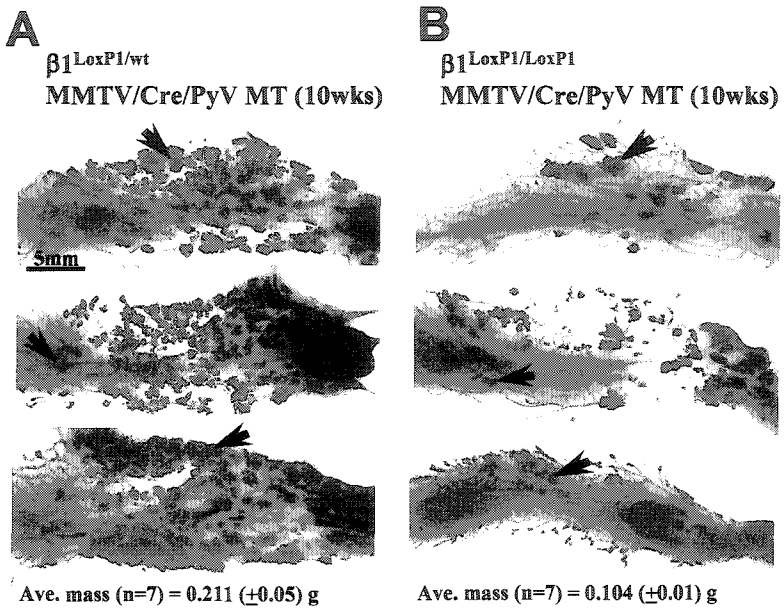

IHC: anti-β1-integrin
MMTV/PyV MT (10wk)
100μm
MMTV/PyV MT (10wk)
25μm

Figure 3.2.3.2 Mammary gland tumourigenesis is impaired in $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT animals

Mice expressing the PyV MT antigen normally develop multiple neoplastic mammary gland lesions by the age of 10 weeks (**A**). In the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT background, however, the number of these lesions is dramatically reduced (**B**). Representative lesions are indicated by arrows. The average mass of seven glands from each genotype is indicated below the bottom panels.


A
β1LoxP1/wt
MMTV/Cre/PyV MT (10wks)
5mm
Ave. mass (n=7) = 0.211 (±0.05) g
B
β1LoxP1/LoxP1
MMTV/Cre/PyV MT (10wks)
Ave. mass (n=7) = 0.104 (±0.01) g

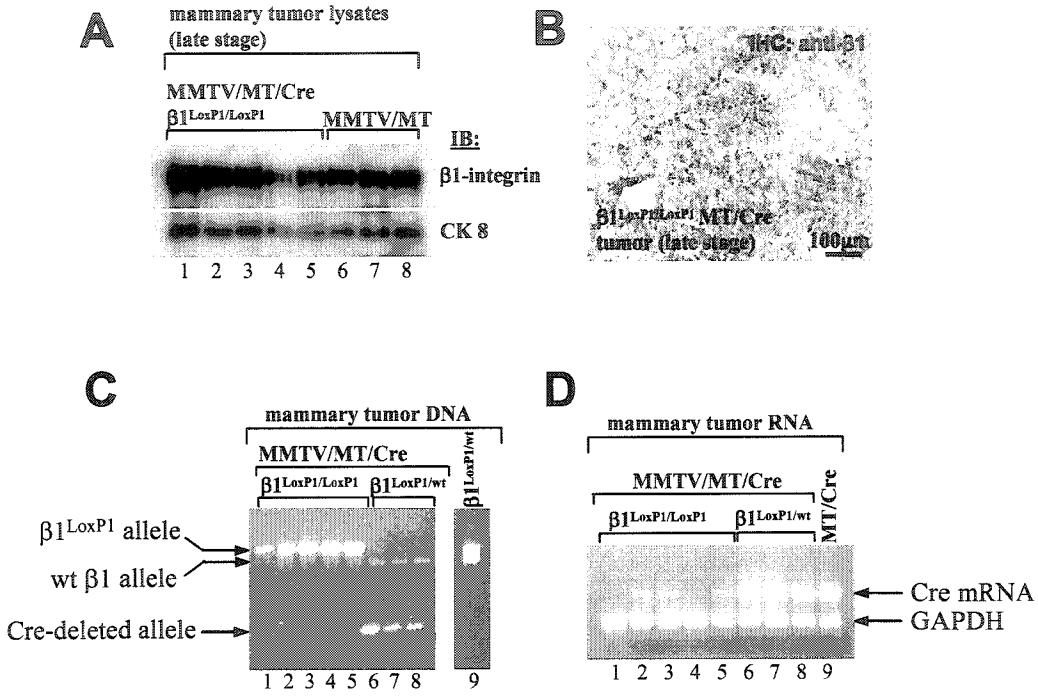
3.2.4 Tumours arising in $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT mice undergo selection for β1-integrin expression

The results described in Section 3.2.3 above suggest that the loss of β1-integrin expression in $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT mice impairs the overall rate of mammary gland transformation by the PyV MT oncoprotein. Eventually, however, solid mammary tumours appeared in these animals, growing at a rate comparable to those of control mice. Given the stochastic expression pattern of the MMTV/Cre transgene (Figure 3.2.2.2B), it is conceivable that these tumours were arising from cells which did not express the MMTV/Cre transgene, and which therefore maintained expression of β1-integrin.

In order to test this hypothesis, mammary tumours from $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT and $\beta1^{LoxP1/wt}$ MMTV/Cre/PyV MT control mice were subjected to molecular and immunohistochemical analyses, to determine the state of β1-integrin expression. As shown by the immunoblot analysis in Figure 3.2.4A, β1-integrin protein is indeed expressed in tumours derived from $\beta1^{LoxP1/LoxP1}$ MMTV/cre/PyV MT mice, at levels comparable to those of control animals (compare lanes 1-5 with lanes 6-8). In addition, immunohistochemical analysis of sections prepared from these tumours revealed uniform expression of β1-integrin protein throughout the tumour tissue (Figure 3.2.4B). Maintenance of β1-integrin protein expression in these MMTV/cre/PyV MT-derived tumours was found to be consistent with the near absence of Cre-induced excision of the $\beta1^{LoxP1}$ allele, as determined by PCR amplification of tumour-derived DNA (Figure 3.2.4C, lanes 1-5). The $\beta1^{LoxP1}$ allele, by contrast, was efficiently excised in tumours from $\beta1^{LoxP1/wt}$ MMTV/cre/PyV MT mice, which harbor 1 copy of the wild-type β1-integrin allele (Figure 3.2.4C, lanes 6-8). Importantly, the difference in the extent of excision of the $\beta1^{LoxP1}$ allele between the $\beta1^{LoxP1/LoxP1}$ MMTV/cre/PyV MT and $\beta1^{LoxP1/wt}$ MMTV/cre/PyV MT mice was found to correlate with levels of Cre expression in these tumours, as determined by RT-PCR analysis of tumour-derived RNA (Figure

Figure 3.2.4 Expression of β1-integrin is maintained in $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT–derived tumours

Tumours arising in $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT mice are populated by cells maintaining expression of β1-integrin protein, as shown by immunoblot (**A**) and immunohistochemical anaylsis (**B**) of $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT–derived tumours, using an anti-β1-integrin antibody. The retention of β1-integrin expression is consistent with an observed lack of Cre-mediated excision in these tumors (**C**), which is correlated with low levels of Cre expression (**D**).


A
mammary tumor lysates
(late stage)
MMTV/MT/Cre
β1LoxP1/LoxP1
MMTV/MT
IB:
β1-integrin
CK 8
1 2 3 4 5 6 7 8
B
IHC: anti-β1
β1LoxP1/LoxP1 MT/Cre
tumor (late stage)
100μm
C
mammary tumor DNA
MMTV/MT/Cre
β1LoxP1/LoxP1
β1LoxP1/wt
β1LoxP1/wt
β1LoxP1 allele
wt β1 allele
Cre-deleted allele
1 2 3 4 5 6 7 8
9
D
mammary tumor RNA
MMTV/MT/Cre
β1LoxP1/LoxP1
β1LoxP1/wt
MT/Cre
Cre mRNA
GAPDH
1 2 3 4 5 6 7 8 9

3.2.4D, compare lanes 1-5 with lanes 6-8). These results therefore support the hypothesis that tumours arising in the $\beta1^{LoxP1/LoxP1}$MMTV/Cre/PyV MT mice are populated by cells retaining expression of β1-integrin, due to the absence of MMTV/Cre expression and Cre-mediated excision in those cells.

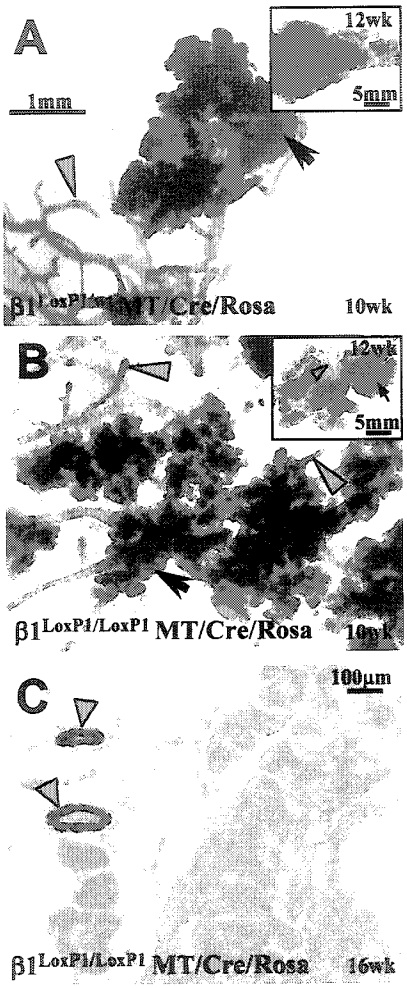
3.2.5 Cre-expressing cells do not contribute to tumourigenesis in the $\beta1^{LoxP1/LoxP1}$ MMTV/cre/PyV MT background

The observation that β1-integrin expression is maintained in $\beta1^{LoxP1/LoxP1}$ MMTV/cre/PyV MT-derived mammary tumours suggests that cells expressing the MMTV/Cre transgene are refractory to transformation in this genetic background. To test this hypothesis, the GTRosa26 reporter construct was introduced into the $\beta1^{LoxP1/LoxP1}$MMTV/Cre/PyV MT and $\beta1^{LoxP1/wt}$ MMTV/Cre/PyV MT mice, in order to compare the fate of Cre-expressing cells during PyV MT-induced transformation in these two sets of animals. After obtaining the desired genetic combinations, mammary glands were removed from 10 to 12 week-old GTRosa26 $\beta1^{LoxP1/wt}$MMTV/Cre/PyV MT control mice, as well as from age-matched mice of the GTRosa26 $\beta1^{LoxP1/LoxP1}$MMTV/Cre/PyV MT background. The glands were then stained with Xgal and mounted, in order to visualize the distribution of Cre-expressing cells *in situ*. Consistent with the results of the RT-PCR analysis shown in Figure 3.2.4D, Cre-expressing cells in the GTRosa26 $\beta1^{LoxP1/wt}$ MMTV/Cre/PyV MT control background were present in both the hyperplastic lesions (Figure 3.2.5A, black arrow) and normal ductal structures (Figure 3.2.5A, green arrowhead) of the glands, suggesting that they were not subject to a selective disadvantage during tumorigenesis. The ability of Cre-expressing cells to form tumours in these mice is even more apparent by the robust staining seen throughout the gland of a 12 week-old tumour-bearing animal (Figure 3.2.5A, inset).

In the GTRosa26 $\beta1^{LoxP1/LoxP1}$MMTV/Cre/PyV MT background, however, Cre-expressing cells were found to be located exclusively within the normal ductal structures (Figure 3.2.5B and inset, green arrowheads). Regions of the glands undergoing

Figure 3.2.5 Cre-expressing cells do not undergo transformation in the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT background

The fate of Cre-expressing cells in $\beta1^{LoxP1/wt}$ MMTV/Cre/PyV MT and $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT mice were compared by introducing the Cre-responsive GTRosa26 reporter construct. Mammary glands from both combinations were stained *in situ* with Xgal, in order to identify the Cre-expressing cells. In the $\beta1^{LoxP1/wt}$ MMTV/Cre/PyV MT combination (**A**), Cre-expressing cells (blue) are present in both normal (green arrowhead) and neoplastic (black arrow) tissue. The inset shows staining for Cre-induced β-galactosidase activity (blue) throughout the tumour-filled gland of one of these mice. In the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT background, however (**B, C**), Cre-expressing cells (blue) are present only in the morphologically normal regions of the gland (green arrowheads).


A
12wk
5mm
1mm
β1LoxP1/LoxP1 MT/Cre/Rosa
10wk
B
12wk
5mm
β1LoxP1/LoxP1 MT/Cre/Rosa
10wk
100μm
C
β1LoxP1/LoxP1 MT/Cre/Rosa
16wk

hyperplastic proliferation, by contrast, showed no evidence of Cre-mediated β-galactosidase activity (Figure 3.2.5B and inset, black arrows). This pattern of staining was also observed in sections of mammary tumours from these mice (Figure 3.2.5C), where morphologically normal, β-galactosidase-positive ductal structures (green arrowheads) could be seen running through β-galactosidase-negative regions of tumour (pink).

Cells expressing the MMTV/Cre transgene, therefore, appear to be refractory to PyV MT-induced transformation in the $\beta1^{LoxP1/LoxP1}$MMTV/Cre/PyV MT background. Since these mice contain 2 copies of the $\beta1^{LoxP1}$ allele in their genome, these results suggest that a functional copy of the β1-integrin gene is required for oncogenic transformation of individual epithelial cells in the MMTV/PyV MT mice.

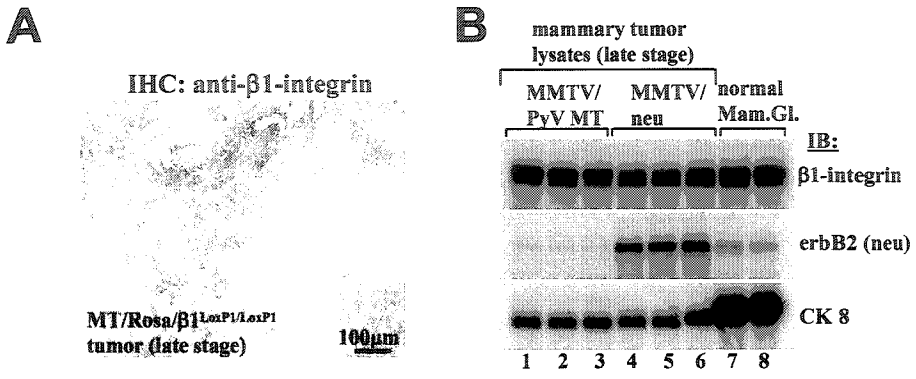
3.2.6 β1-integrin expression is required for maintenance of the tumour cell phenotype

The results presented thus far strongly suggest that β1-integrin expression is necessary for the early stages of PyV MT-induced transformation *in vivo*. While these results are consistent with the distribution of β1-integrin protein in early PyV MT-induced mammary lesions (see Figure 3.2.3.1), it is important to note that β1-integrin expression is maintained throughout mammary tumour progression in the MMTV/PyV MT mice. This conclusion is based on immunohistochemical (Figure 3.2.6.1A) and immunoblot (Figure 3.2.6.1B, lanes 1-3) analysis of late-stage tumours derived from MMTV/PyV MT animals immediately prior to end-point (4-5 months).

In order to determine, therefore, if β1-integrin expression is also required for maintaining the growth of PyV MT-induced tumours, a strategy was devised to excise the $\beta1^{LoxP1}$ alleles from $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT mice *ex vivo* (Figure 3.2.6.2A). This approach first involved the culture of primary tumour cells from $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumours, as well as from tumours arising in $\beta1^{LoxP1/wt}$ MMTV/PyV MT control animals. Once these cultures were established, excision of the

Figure 3.2.6.1 β1-integrin protein expression is maintained during the growth of PyV MT-induced mammary tumours

Immunohistochemical analysis of tumour sections from end-point MMTV/PyV MT mice, using an anit-β1-integrin antibody, reveals expression of β1-integrin protein throughout the tumour tissue (**A**). Expression of β1-integrin protein was confirmed in these tissue by immunoblot analysis of tumour lysates (**B**, lanes 1-3). β1-integrin protein is also expressed in MMTV/erbB2-derived tumours (**B**, lanes 4-6), suggesting that expression of β1-integrin may be common to different oncogene-induced tumour types.

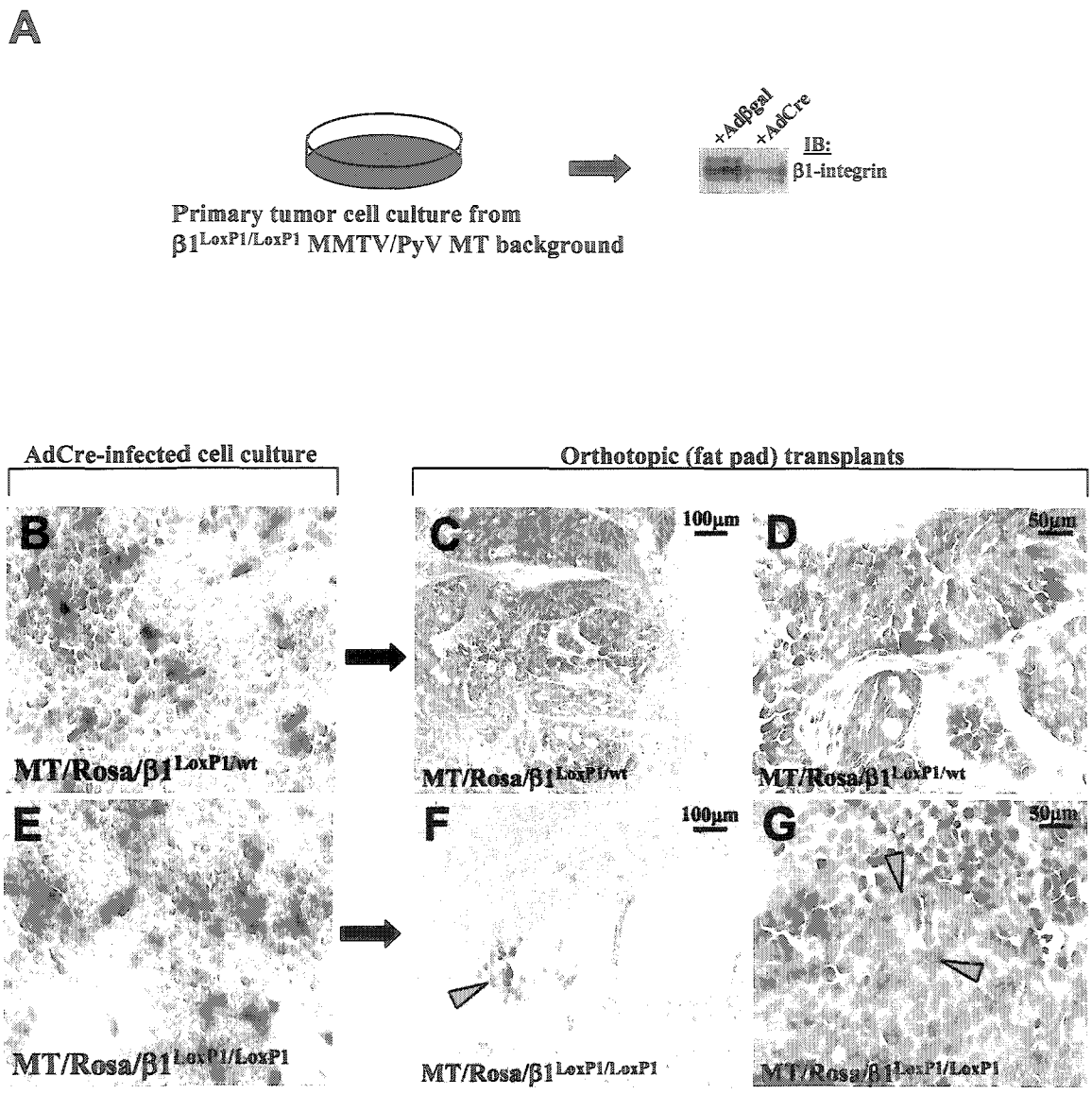

A
IHC: anti-β1-integrin
MT/Rosa/β1LoxP1/LoxP1
tumor (late stage)
100μm
B
mammary tumor
lysates (late stage)
MMTV/
PyV MT
MMTV/
neu
normal
Mam.Gl.
IB:
β1-integrin
erbB2 (neu)
CK 8
1 2 3 4 5 6 7 8

$\beta1^{LoxP1}$ alleles could be induced by infection with an adenovirus vector expressing the Cre recombinase (AdCre). As shown by the immunoblot analysis presented in Figure 3.2.6.2A, infection with AdCre results in a reduction in β1-integrin protein levels in $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumour cells, relative to cells infected with a control virus (Adβgal). Inclusion of the GTRosa26 reporter in the donor mice of each strain facilitates colourimetric identification of individual cells undergoing Cre-mediated excision using this approach (Figure 3.2.6.2B and 3.2.6.2E).

The tumourigenic capacity of AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT cells was then determined by injecting cells from the infected culture into the cleared fat pads of 10 syngeneic FVB recipients. As a control, cells from the AdCre-infected $\beta1^{LoxP1/wt}$ MMTV/PyV MT culture were injected contralaterally into those same mice. After 3 to 4 weeks, solid tumours could be palpated at the site of injection in 5 mice of the control group, and 7 mice of the AdCre-infected GTRosa26 $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT culture. In order to determine if Cre-expressing cells from either culture were able to contribute equally to the development of the tumours, each tumour was stained for β-galactosidase activity, and then sectioned for microscopic examination (Figure 3.2.6.2, panels C,D,F and G). As expected, tumours derived from $\beta1^{LoxP1/wt}$ cells exhibited robust staining for β-galactosidase activation, indicating that mammary tumour cells retaining one functional copy of the β1-integrin gene were not impaired in their ability to contribute to the tumour mass (Figures 3.2.6.2C and 3.2.6.2D). In contrast, very few β-galactosidase-positive tumour cells were detected in animals transplanted with infected cells from the $\beta1^{LoxP1/LoxP1}$ background (Figures 3.2.6.2F and 3.2.6.2G). The small patches of Cre-expressing cells present in these tumours, rather, seemed to delineate the site of inoculation (Figure 3.2.6.2F, green arrowhead). At higher magnification (Figure 3.2.6.2G), these Cre-expressing cells could be identified as single, isolated entities, showing no evidence of having undergone proliferation during growth of the tumour (green arrowheads). These results, therefore, suggest that β1-integrin expression is required for maintenance of the tumourigenic phenotype of PyV MT-transformed cells.

Figure 3.2.6.2 Late-stage tumour cells lacking β1-integrin fail to proliferate *in vivo*

To determine if the tumourigenic phenotype of MMTV/PyV MT-derived tumour cells requires β1-integrin expression, a strategy was devised to ablate β1-integrin expression from tumour cells *ex vivo* (A). This approach involves AdCre infection of tumour cells cultured from $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT mice, which results in reduced levels of β1-integrin protein in the infected cultures (see immunoblot in A). Inclusion of the GTRosa26 reporter facilitates identification of Cre-expressing cells in AdCre-infected cultures of both $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT (**B**) and $\beta1^{LoxP1/wt}$ MMTV/PyV MT-derived (**E**) tumour cells. The ability of AdCre-infected cells from either culture to form tumours *in vivo* can be determined by fat pad transplantation of the infected cultures, followed by Xgal-staining of tumours arising at the sites of injection (**C,D,F,G**).

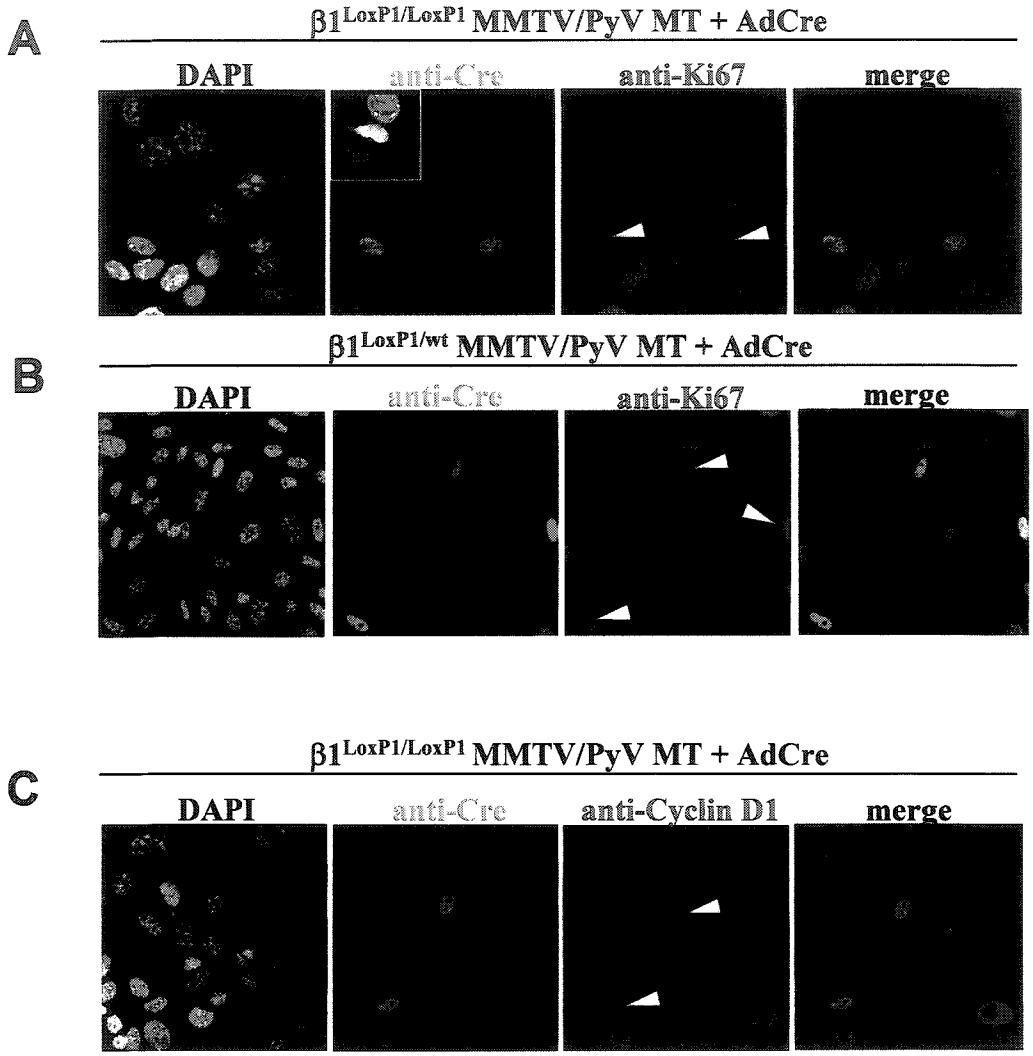

A
+Adβgal
+AdCre
IB:
β1-integrin
Primary tumor cell culture from
β1LoxP1/LoxP1 MMTV/PyV MT background
AdCre-infected cell culture
Orthotopic (fat pad) transplants
B
MT/Rosa/β1LoxP1/wt
C
100μm
MT/Rosa/β1LoxP1/wt
D
50μm
MT/Rosa/β1LoxP1/wt
E
MT/Rosa/β1LoxP1/LoxP1
F
100μm
MT/Rosa/β1LoxP1/LoxP1
G
50μm
MT/Rosa/β1LoxP1/LoxP1

3.2.7 $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT tumour cells expressing the Cre recombinase show evidence of a proliferative block

As shown in Figure 3.2.6.2, the tumourigenic capacity of PyV MT-induced tumour cells *in vivo* is blocked following inhibition of β1-integrin expression. Interestingly, the inhibiton of β1-integrin binding activity in cultured human breast cancer cells has previously been shown to result in a reversion of the transformed phenotype through induction of a proliferative block, rather than apoptotic cell death (Weaver et al., 1997). As a result, the proliferative capacity of AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumor cells was measured in culture, to determine whether the results in Section 3.2.6 could also be explained by the induction of a proliferative block. Cells undergoing proliferation in AdCre-infected cultures were identified by immunofluorescence microscopy, using an antibody specifically recognizing the Ki67 nuclear antigen, a marker of cell proliferation (Figure 3.2.7A, third panel). Similarly, cells expressing the Cre recombinase were identified by co-staining the same infected cultures with an anti-Cre antibody (Figure 3.2.7A, second panel). As shown in Figure 3.2.7A, the expression of Cre and Ki67 were found to be mutually exclusive in the AdCre-infected cultures of $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumour cells, which was confirmed by the analysis of multiple fields (Figure 3.2.7D). The Cre and Ki67 antigens, however, were found to be co-expressed in a large proportion of AdCre-infected $\beta1^{LoxP1/wt}$ MMTV/PyV MT-derived control cells (Figures 3.2.7B and 3.2.7D). Since $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumour cells differ from control cells only with respect to 2 copies of the $\beta1^{LoxP1}$ allele, these results suggest that β1-integrin expression is required for proliferation of the PyV MT-transformed tumour cells under these culture conditions. The exclusion of cyclin D1 from the nuclei of AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumour cells, as shown by co-immunostaining with anit-Cre and anti-cyclin D1 antibodies (Figures 3.2.7C and 3.2.7E),

Figure 3.2.7 AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumour cells fail to express markers of cell proliferation and cell cycle progression

Cultures prespared from $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumours (**A** and **C**), as well as $\beta1^{LoxP1/wt}$ MMTV/PyV MT-derived control cells (**B**), were infected with AdCre. Cre-expressing cells were identified by immunofluorescence microscopy, using an anti-Cre primary antibody and a FITC-labeled secondary antibody (green). Functional excision in Cre-expressing cells was confirmed by co-incubation with an anti-β-galactosidase antibody and a Cy3-labeled secondary antibody (red) (inset in **A**). Proliferating cells were identified in **A** and **B** using an antibody to the Ki67 marker of proliferation (red). (**D**) Quantitative analysis of Cre and Ki67 co-localization. (**C**) An anti-cyclin D1 antibody and Cy3-labeled secondary antibody (red) was used to identify cells expressing nuclear cyclin D1 protein. (**E**) Quantitative analysis of nuclear Cre and cyclin D1 co-localization.


A
β1LoxP1/LoxP1 MMTV/PyV MT + AdCre
DAPI
anti-Cre
anti-Ki67
merge
B
β1LoxP1/wt MMTV/PyV MT + AdCre
DAPI
anti-Cre
anti-Ki67
merge
C
β1LoxP1/LoxP1 MMTV/PyV MT + AdCre
DAPI
anti-Cre
anti-Cyclin D1
merge
D
% Ki67-positive cells
59/95
11/35
23/80
Cre-pos
Cre-neg
Cre-pos
Cre-neg
LoxP1/LoxP1
LoxP1/wt
E
% cyclin D1-positive cells
39/53
0/32
Cre-pos
Cre-neg

further supports this hypothesis by demonstrating that these cells fail to show evidence of undergoing cell cycle progression.

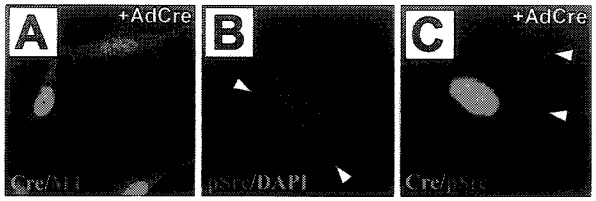
3.2.8 Excision of β1-integrin suppresses the tyrosine phosphorylation of FAK in PyV MT-transformed tumour cells

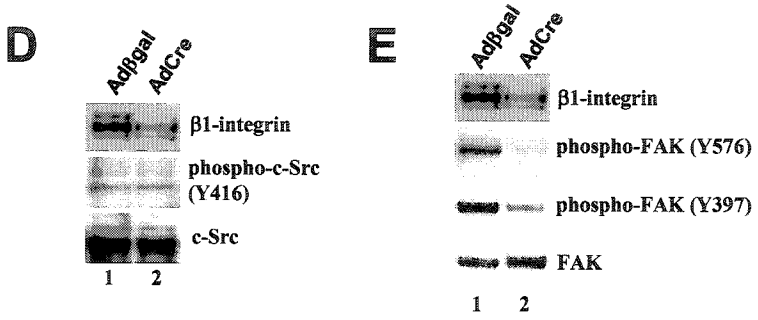
The results presented in Section 3.2.7 are consistent with a role for β1-integrin in promoting cell cycle progression (Assoian and Schwartz, 2001; Schwartz and Assoian, 2001). This property of β1-integrin signaling has been attributed primarily to the activation of cyclin D1 expression through the c-Src-mediated phosphorylation of FAK (Oktay et al., 1999). Since PyV MT is a potent activator of c-Src kinase activity (Ichaso and Dilworth, 2001), the status of both c-Src activation and PyV MT expression was examined in AdCre-infected PyV MT-induced tumor cells homozygous for the $\beta1^{LoxP1}$ allele. Since c-Src activity has previously been shown to be necessary for PyV MT-induced transformation of the mouse mammary gland (Guy et al., 1994), the attenuation of either c-Src activity or PyV MT expression may provide a possible explanation for the inhibition of proliferation in the AdCre-infected cells.

As shown by immunofluorescent microscopic analysis of AdCre-infected cells, the expression of Cre in the $\beta1^{LoxP1/LoxP1}$ background did not inhibit expression of the PyV MT antigen (Figure 3.2.8.1A). Similarly, infection of these cells did not alter the expression or distribution of the activated form of c-Src, as determined using phosphospecific antibodies directed to tyrosine 416 (Y416) in the activation domain of the c-Src molecule (compare Figures 3.2.8.1B and 3.2.8.1C, white arrowheads). Maintenance of c-Src phosphorylation in these cells was confirmed by immunoblot analysis of protein lysates, using the same phospho-specific antibody (Figure 3.2.8.1D). In spite of reduced levels of β1-integrin protein (Figure 3.2.8.1D, top panel), this analysis revealed comparable levels of Y416 phosphorylation between Adβgal and AdCre-infected cells (Figure 3.2.8.1D, middle panel). These results suggest that the suppression

Figure 3.2.8.1 Phosphorylation of FAK is suppressed in AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumour cells

Cultures from $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumours were incubated with anti-Cre antibody, and either anti-PyV MT (**A**) or anti-phospho-c-Src (Y416) (**B**, **C**) antibodies. Neither the expression of PyV MT (red staining in **A**) nor the distribution of p-c-Src (red staining in **B** and **C**) were altered by expression of Cre (green) in these cells. (**D**) Immunoblot analysis of c-Src phosphorylation in Adβgal (lane 1) versus AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumour cells, using antibodies to phospho-c-Src (Y416) and c-Src. (**E**) Immunoblot analysis of FAK phosphorylation in Adβgal (lane 1) versus AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumour cells. The phosphorylation status of two tyrosine residues (Y397 and Y576) were examined using anti-phosphotyrosine antibodies specific for these residues. (**F**) Focal adhesion formation was confirmed in AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived cells using an anti-paxillin antibody (red). The localization of FAK in uninfected cells (**G**) was compared to AdCre-infected cells (**H**), using antibodies specific for FAK (red) and Cre (green).


A
+AdCre
B
C
+AdCre


D
Adβgal
AdCre
β1-integrin
phospho-c-Src
(Y416)
c-Src
1
2
E
Adβgal
AdCre
β1-integrin
phospho-FAK (Y576)
phospho-FAK (Y397)
FAK
1
2


F
+AdCre
G
H
+AdCre

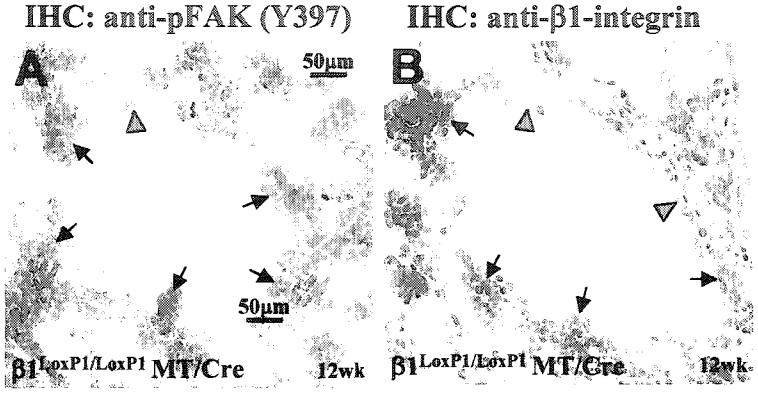
of proliferation in AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumour cells was not due to either the attenuation of PyV MT expression nor the inhibition of PyV MT signaling through the c-Src tyrosine kinase.

Since FAK has been shown to be important for mediating the biological effects of both c-Src and β1-integrin (Gabarra-Niecko et al., 2003; Guan, 1997), including the c-Src-mediated induction of cell proliferation (Oktay et al., 1999; Zhao et al., 1998), the phosphorylation status of FAK was examined next in AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived cells. In response to integrin engagement, FAK undergoes phosphorylation at tyrosine residue 576 (Y576) in its kinase domain, followed by autophosphorylation at the c-Src binding site (Y397) (Kornberg et al., 1992; Shen and Schaller, 1999). The phosphorylation of additional tyrosine residues by c-Src subsequently provides binding sites for signaling molecules such as Grb2, important for the regulation of cell cycle progression (Oktay et al., 1999). Using phospho-specific antibodies, the phosphorylation status of the Y327 and Y576 FAK residues was determined by immunoblot analysis of AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumor cell lysates. As shown in Figure 3.2.8.1E, phosphorylation of both Y576 and Y397 on FAK were indeed found to be reduced in AdCre infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT cells (Figure 3.2.8.1E, lane 2), as compared to Adβgal-infected cells (Figure 3.2.8.1E, lane 1). Although the targeted disruption of β1-integrin in these cells did not interfere with the formation of paxillin-containing focal adhesion sites (Figure 3.2.8.1F, white arrowheads), there was a corresponding reduction in total FAK protein within these peripheral focal contacts following AdCre-infection (compare Figure 3.2.8.1G to Figure 3.2.8.1H). Taken together, these observations suggest that the ablation of β1-integrin results in reduced levels of FAK phosphorylation and a corresponding failure to recruit FAK to focal adhesion contacts. Given that FAK is an important downstream target of c-Src during the induction of cell proliferation and transformation, it is conceivable that the suppression of FAK phosphorylation, and its absence in focal adhesion sites, may account for the observed block in cell proliferation in AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumour cells.

Since these results suggest that FAK phosphorylation is critical for PyV MT-induced cell cycle progression and transformation, the distribution of phospho-FAK in mammary glands of $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT mice was examined by immunohistochemistry, using an antibody specific for phosphotyrosine 397. As shown by the staining pattern in Figure 3.2.8.2A, phospho-FAK (Y397) was found to be clearly visible in regions of the glands undergoing hyperplastic proliferation (black arrows), as compared to non-transformed regions of the epithelium (green arrowhead). Since the distribution of phospho-FAK overlaps with the expression of β1-integrin in these glands (compare with Figure 3.2.8.2B), these results argue that β1-integrin-mediated FAK phosphorylation may be necessary for PyV MT-induced mammary tumorigenesis *in vivo*.

Figure 3.2.8.2 The phosphorylation of FAK is correlated with the pattern of β1-integrin expression in MMTV/PyV MT-induced tumors

Sections from $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT-derived tumours were incubated with anti-phospho-FAK (Y397) (A) and anti-β1-integrin (B) antibodies, followed by an HRP-conjugated secondary antibody. Expression of both proteins (brown) is prominent in regions of the mammary epithelium undergoing hyperplastic expansion (black arrows), as opposed to non-transformed regions of the gland (green arrowheads), where expression of p-FAK and β1-integrin is undetectable.


IHC: anti-pFAK (Y397)
IHC: anti-β1-integrin
A
B
50μm
50μm
β1LoxP1/LoxP1 MT/Cre
12wk
β1LoxP1/LoxP1 MT/Cre
12wk

3.3 DISCUSSION

3.3.1 Determining the role of β1-integrin during mammary gland transformation *in vivo*

The experimental results presented in Chapter 3 demonstrate that β1-integrin expression is required for the initial stages of mammary tumourigenesis in MMTV/PyV MT mice, as well as for maintenance of the tumourigenic phenotype in late-stage PyV MT-induced tumours. Whereas earlier studies provided compelling evidence that β1-integrin binding activity was required for the deregulated growth of human breast cancer cell lines in an artificially reconstituted matrix (Wang et al., 2002; Weaver et al., 1997), the results presented in Chapter 3 provide the first direct demonstration of this phenomenon in a physiologically relevant context, helping to resolve an important issue regarding the clinical relevance of β1-integrin expression in human cancers (Shaw, 1999; Zutter et al., 1998).

The conclusions presented in Chapter 3 were based on the use of a conditional, LoxP1-flanked β1-integrin allele, previously shown to be an efficient target of Cre-mediated recombination *in vivo*. As shown in section 3.2.1 of this chapter, the excision of this $\beta1^{LoxP1}$ allele could be targeted specifically to the mouse mammary gland epithelium by expression of an MMTV/Cre transgene. Consistent with the previously published applications of this $\beta1^{LoxP1}$ allele in other murine tissues, the mammary-specific expression of Cre was shown to result in reduced β1-integrin protein levels in the mammary glands of $\beta1^{LoxP1/LoxP1}$ MMTV/Cre mice (Figure 3.2.1). The use of immunohistological techniques to follow the fate of β1-integrin-null cells during tumourigenesis and development, however, was problematic, since the membrane-bound localization of the β1-integrin subunit would make it difficult to distinguish β1-integrin-null cells from their neighbours. This technical limitation was exacerbated by the stochastic nature of the MMTV-LTR promoter (Figure 3.2.2.2). As a result, the Cre-responsive GTRosa26 β-galactosidase reporter construct was introduced into the mice as

a way to accurately identify mammary epithlelial cells undergoing Cre-mediated excision events *in situ*. Comparing the fate of Cre-expressing cells on the $\beta1^{LoxP1/LoxP1}$ versus $\beta1^{LoxP1/wt}$ background therefore provided an accurate means to determine if β1-integrin expression was required for both mammary gland development and tumourigenesis *in vivo* (for example, see Figures 3.2.2.3 and 3.2.5).

When the GTRosa26 reporter was introduced into the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT mice, the Cre-expressing (blue) cells could not be detected in regions of the mammary gland undergoing hyperplastic proliferation (see Figures 3.2.5B and 3.2.5C). These cells, however, were found to be abundant throughout the normal ductal structures in the glands from these mice, demonstrating that they nonetheless remained viable in the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT genetic background. In the control $\beta1^{LoxP1/wt}$ MMTV/Cre/PyV MT animals, by contrast, Cre-expressing cells were abundant in both the normal and transformed regions of the gland (see Figure 3.2.5A). The staining pattern in these control glands confirmed that expression of the MMTV/Cre and MMTV/PyV MT transgenes are not mutually exclusive in this model, and that Cre expression itself does not inhibit transformation of the individual mammary epithelial cells. The inability of Cre-expressing cells to undero transformation in the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT background, therefore, could only be attributed to the presence of two $\beta1^{LoxP1}$ alleles, indicating that β1-integrin expression is required for the initiation of tumourigenesis in the MMTV/PyV MT model.

The observation that β1-integrin expression is required for PyV MT-induced transformation of the mouse mammary gland is quite remarkable, given that the PyV MT antigen has very potent transforming properties *in vivo* (Guy et al., 1992a). Previous experiments designed to inhibit transformation in the MMTV/PyV MT model, through the deletion of PI3-K and Shc binding sites, have resulted in longer tumour latency or reduced metastatic burden, but not in complete ablation of transformation (Webster et al., 1998). Only when the MMTV/PyV MT transgene was introduced into a c-Src-null background was there a dramatic effect on PyV MT-induced transformation (Guy et al., 1994).

The phosphorylation and localization of c-Src, however, was not altered in $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumour cells infected with the AdCre vector (Figure 3.2.8.1C). Further analysis of these cells, however, revealed a dramatic reduction in the phosphorylation of FAK, which is both an important target of c-Src kinase activity and a mediator of the biological properties of the c-Src molecule (Figure 3.2.8.1E) (Calalb et al., 1995; Gabarra-Niecko et al., 2003; Xing et al., 1994). Interestingly, many of the cell signaling properties of β1-integrin, including the promotion of cell cycle progression through molecular cross talk with growth factor receptors, have been shown to require c-Src-mediated phosphorylation of the FAK molecule (Schwartz and Assoian, 2001; Zhao et al., 1998). In this regard, phosphorylation of the c-Src binding site (Y397) on the amino terminus of FAK has been shown to be a minimum requirement for the induction of cell proliferation by β1-integrin-associated FAK (Zhao et al., 1998). Given that Y397 is underphosphorylated in AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived cells, these results would argue that the suppression of FAK phosphorylation in β1-integrin-null mammary epithelial cells plays an important role in rendering these cells resistant to PyV MT-induced transformation.

The merit of this hypothesis is strengthened by the results of several recent experiments involving the manipulation of FAK in mouse models as well as human carcinoma cells. In one of these experiments, mice harbouring only one allele of the FAK gene were found to be resistant to DMBA-induced papilloma formation, suggesting that sufficient levels of FAK protein is a critical determinant for carcinogen-induced transformation of the epidermis (McLean et al., 2001). Interestingly, any DMBA-induced nodules arising in this $FAK^{+/-}$ background showed elevated expression from the remaining FAK allele. This phenomenon bears remarkable resemblance to the process of selection for β1-integrin expression during transformation of the mammary gland epithelium in $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT mice, suggesting an overlapping requirement for these molecules during tumourigenesis. In another set of experiments, the suppression of FAK phosphorylation in human hepatocellular carcinoma cells was shown to result in a proliferative block *in vivo*, inducing a state resembling that of tumour

dormancy (Aguirre Ghiso, 2002; Liu et al., 2002). In this case, FAK phosphorylation was inhibited by blocking expression of the uPA receptor, which has been shown to induce phosphorylation of FAK by forming a complex with β1-integrin (Liu et al., 2002). In a third experiment, another group showed that the suppression of FAK phosphorylation in human mammary epithelial cells induced a permanent state of differentiation, rendering them resistant to transformation in a 3-dimensional cell culture environment (Wozniak et al., 2003). The combined results of these experiments provide further evidence that the inhibition of FAK phosphorylation in β1-integrin-null cells may explain both the inhibition of tumourigenesis in the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT mice, as well as the proliferative block in AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumour cells. It is worthy to note that the role of FAK in c-Src-induced transformation has also been demonstrated by manipulation of an immediate downstream effector of FAK signaling, where cells from $p130^{CAS}$-null mice were shown to be refractory to transformation by the activated viral homologue (v-Src) of c-Src (Honda et al., 1998).

The failure to recruit and phosphorylate FAK in β1-integrin-null cells implies that the β1-integrin subunit plays a prominent role in this process in mammary epithelial cells. Although mammary epithelial cells also express the β4-integrin subunit, there are no published reports demonstrating a direct association between β4-integrin and FAK in these cells. A functional interaction between FAK and the β1-integrin subunit, on the other hand, has been well established. Since the phosphorylation of FAK occurs concommitently with localization to focal adhesion sites (Burridge et al., 1992; Kornberg et al., 1992; Shen and Schaller, 1999), the failure to recruit FAK to focal contacts (see Figure 3.2.8H) may explain the reduced levels of FAK autophosphorylation in AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumour cells. The biological impact of this phenomenon has been demonstrated in experiments where expression of dominant-negative versions of FAK, specifically lacking the focal adhesion targeting motif, resulted in decreased DNA synthesis and BrdU incorporation in cultured cells (Gilmore and Romer, 1996; Zhao et al., 1998). These results are indeed consistent with the

proliferative block seen in the AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived cultures (Figure 3.2.7).

3.3.2 β1-integrin expression is not required for normal mammary gland development during puberty

The results of these experiments strongly suggest that the expression of β1-integrin is required for mammary tumourigenesis in the MMTV/PyV MT model. Expression of the β1-integrin subunit, however, was not required for normal mammary gland development in these mice (see Figure 3.2.2.1). As discussed in the introduction to this chapter, this result contradicts the observations reported in a related experiment designed to test the role of β1-integrin during mammary gland development (Klinowska et al., 1999). Following the implantation of beads coated with inhibitory anti-β1-integrin antibodies into the mammary gland fat pad, the authors reported a modest impairment in TEB numbers and ductal migration during puberty (Klinowska et al., 1999). Their approach, however, failed to discriminate between the role of β1-integrin in the myoepithelial versus luminal epithelial population. Given that the β1-integrin subunit is expressed in both the luminal and myoepithelial cell layers (Deugnier et al., 1995; Taddei et al., 2003), it is conceivable that the impaired developmental phenotype reported in the antibody-based experiment was due to a disruption in the myoepithelial contribution to mammary gland outgrowth. Indeed, the implantation of beads coated with inhibitory antibodies into the fat pad may block the contribution of a variety of cell types, including those of the mammary gland vasculature.

A developmental role for β1-integrin was also suggested by the results of various cell culture-based experiments, demonstrating that normal luminal epithelial cell physiology requires adhesion through the β1-integrin subunit (Farrelly et al., 1999; Streuli et al., 1995). The discrepancy between these *in vitro* results and those involving the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre mice, however, may reflect on the experimental protocol used in the cell culture-based experiments, where cells were prepared from lactating

animals and cultured in the presence of lactogenic hormones (Farrelly et al., 1999; Streuli et al., 1995). As a result, it is conceivable that the results of the cell culture experiments reflect a role for β1-integrin during lactation, rather than in the virgin state. Consistent with this hypothesis, expression of an MMTV-driven dominant-negative β1-integrin allele, consisting of the β1-integrin cytoplasmic domain fused to the CD4 molecule ectodomain (MMTV/β1-cyto), resulted in a modest impairment of mammary gland development during lactation, while normal mammary gland outgrowth was not impaired in virgin animals (Faraldo et al., 1998; Faraldo et al., 2000). When taken together, the observations of Faraldo et al. (1998; 2000) and the results described in Chapter 3 of this thesis provide important demonstrations *in vivo* that luminal epithelial-specific expression of β1-integrin is not required for normal mammary gland development in the mouse.

CHAPTER 4

Overexpression of ILK in the mouse mammary gland epithelium results in a hyperplastic mammary gland phenotype and mammary gland tumourigenesis

4.1 INTRODUCTION

As discussed in Chapter 1, ligand-bound integrin receptors play an important role in the activation of signaling pathways involved in cell survival and cell cycle progession (Schwartz and Assoian, 2001). The activation of these pathways involves the assembly of large signaling complexes at the carboxy termini of clustered integrins, as well as lateral signaling through the growth factor receptors. Mutations which affect the signaling properties of these cytoplasmic effector molecules may result in the deregulation of integrin-mediated signaling pathways and the subsequent loss of integrin-mediated control over epithelial cell proliferation and survival. In some cases, the overexpression or activation of integrin-associated kinases contribute to the growth and dissemination of epithelial tumours such as those of the breast (Gabarra-Niecko et al., 2003). The potent transforming properties and tumourigenic roles of activated forms of Ras and c-Src have been particularly well documented in this regard. In addition, the upregulation of FAK has been found to be correlated with aggressive disease (Cance et al., 2000; Gabarra-Niecko et al., 2003; Oktay et al., 2003; Owens et al., 1995), and experiments designed to inhibit FAK expression and activity have revealed important tumourigenic roles for this protein both *in vitro* and *in vivo* (Aguirre Ghiso, 2002; McLean et al., 2001).

By comparison, little is known of the integrin-associated ILK with regards to playing a role in human malignant disease. The 59kDa ILK, which binds to the cytoplasmic domain of the β1-integrin subunit, has been shown to be an important

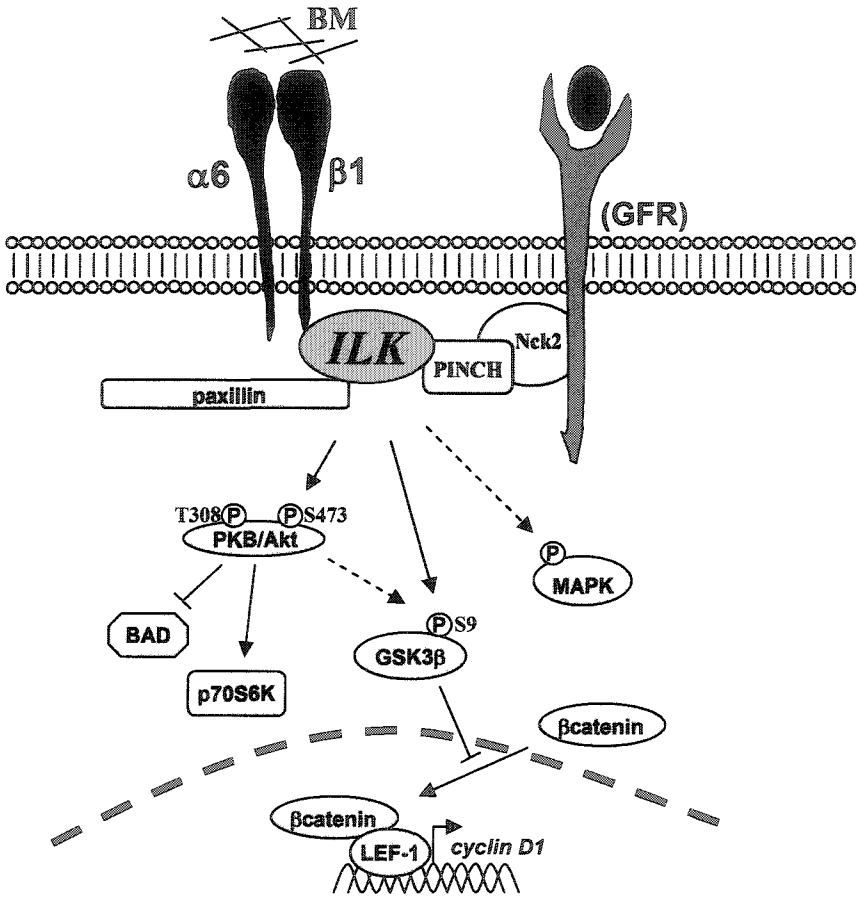
effector of integrin-mediated signaling, as well as regulating the integrin receptor binding properties (Hannigan et al., 1996). Integrin-mediated activation of ILK is PI3-K-dependent, and ILK has been shown to be an important component of PI3-K-induced cell survival pathways in cultured cells (Delcommenne et al., 1998; Troussard et al., 1999). A role for ILK in PI3-K-mediated cell survival has been attributed specifically to the ILK-induced phosphorylation and activation of the anti-apoptotic PKB/Akt kinase (Delcommenne et al., 1998; Atwell et al., 2000). Cells overexpressing ILK are indeed resistant to a form of apoptosis (anoikis) when forced into suspension (Attwell et al., 2000).

Overexpression of ILK in cultured epithelial cells also results in the phosphorylation of glycogen synthase kinase-3β (GSK-3β), a negative regulator of the Wnt—β-catenin signaling pathway (Delcommenne et al., 1998). GSK-3β induces ubiquitination and proteasome-mediated degradation of β-catenin through the phosphorylation of β-catenin in complex with axin and the adenomatous polyposis coli (APC) gene product (Aberle et al., 1997; Kishida et al., 1998). Phosphorylation of serine residue 9 on GSK-3β by ILK results in inhibition of GSK-3β kinase activity, resulting in the stabilization of β-catenin and activation of the β-catenin/LEF-1 transcription complex (Delcommenne et al., 1998; Novak et al., 1998; Troussard et al., 1999). Upregulation of β-catenin/LEF-1 activity, following loss of the APC locus, is indeed associated with the progression of human colon carcinoma (Korinek et al., 1997; Morin et al., 1997). Interestingly, ILK expression and kinase activity have been found to be deregulated in colon cancer samples (Marotta et al., 2001), suggesting a potential role for ILK in the progression of this disease, possibly through the activation of the β-catenin/LEF-1 transcription factor complex (a summary of ILK signaling properties is presented in Figure 4.1).

Cultured epithelial cells overexpressing ILK exhibit elevated levels of MAPK phosphorylation, in addition to anchorage-independent cell cycle progression through transcriptional upregulation of the cyclin D1 gene (D'Amico et al., 2000; Huang et al., 2000; Radeva et al., 1997). These cells are tumourigenic in immunocompromised

Figure 4.1 ILK is a β1-integrin-associated effector of cell proliferation and survival pathways

Several important cell signaling molecules are phosphorylated in response to ILK overexpression in cultured epithelial cells. In addition, inhibition of ILK kinase activity can block the phosphorylation of these targets in response to cell adhesion, demonstrating the role of ILK as an effector of β1-integrin signaling. By directly phosphorylating serine residue 473 (S473) of PKB/Akt, ILK has been shown to activate the serine/threonine kinase activity of this molecule. Activated PKB/Akt induces both cell survival and translational machinery, through the inhibition of the pro-apoptotic BAD and the phosphorylation of p70S6 kinase (p70S6K). The inhibitor of the Wnt signaling pathway, GSK-3β, has also been shown to be a target of ILK kinase activity. Either directly or indirectly through PKB/Akt, ILK induces phosphorylation of GSK-3β on serine residue 9 (S9). Phosphorylation of this residue inhibits the ability of GSK-3β to induced ubiquitin-mediated degradation of β-catenin, resulting in stabilization of β-catenin and formation of the β-catenin/LEF-1 transcription complex. Cells overexpressing ILK also exhibit an increase in the levels of MAPK phosphorylation. As a result, ILK is believed to play a central role in regulating adhesion-dependent proliferation and survival.


BM
α6
β1
(GFR)
ILK
Nck2
PINCH
paxillin
T308
P
P
S473
PKB/Akt
P
MAPK
BAD
P
S9
GSK3β
p70S6K
βcatenin
βcatenin
LEF-1
cyclin D1

animals, suggesting that ILK may play an oncogenic role when overexpressed in epithelial cells. Importantly, ILK levels have been found to be elevated in human cancers, including ovarian (Ahmed et al., 2003), prostate (Graff et al., 2001), melanoma (Dai et al., 2003; Janji et al., 1999), colon (Marotta et al., 2001), Ewing's sarcoma and neuroectodermal tumours (Chung et al., 1998). In almost all cases, the overexpression of ILK is associated with the transition from benign lesions to aggressive disease (Ahmed et al., 2003; Dai et al., 2003; Graff et al., 2001; Janji et al., 1999). Since invasive cancers often show evidence of an epithelial-to-mesenchymal transition (EMT) (Thiery, 2002), the correlation of ILK expression with the high-grade tumour phenotype may be clinically relevant, given that ILK overexpression has been shown to induce a mesenchymal phenotype in cultured mammary epithelial cells (Somasiri et al., 2001). Recently, ILK has been shown to play an important role in promoting tumour angiogenesis, offering an alternative explanation for the upregulation of ILK in human cancers (Tan et al., 2004).

In summary, the overexpression of ILK in cultured epithelial cells results in changes characteristic of oncogenic transformation. The altered growth properties of these cells result from constitutive cell cycle progression and the activation of cell survival pathways, possibly through the activation of downstream targets such as MAPK, cyclin D1 and PKB/Akt. When combined with the expression pattern of ILK in clinical tumour samples, these observations implicate ILK as a molecule potentially involved in the induction and/or progression of human cancers. Direct evidence of an oncogenic role for ILK in a physiological context, however, has not yet been provided. As a result, a transgenic approach was designed to test the oncogenic potential for ILK overexpression *in vivo*, using the mouse mammary gland epithelium as the target tissue. The results of this experiment are presented in Section 4.2 below.

4.2 RESULTS

4.2.1 Targeted overexpression of ILK in the mammary epithelium

In order to determine if ILK can play an oncogenic role when overexpressed in the mammary epithelium, the full length cDNA for human ILK was placed under the transcriptional control of the MMTV-LTR (Figure 4.2.1A) and microinjected into one-cell mouse zygotes of the FVB strain. To identify founder mice expressing the MMTV/ILK expression cassette, RNA was isolated from the mammary glands of 10 week-old virgin female founder animals and subjected to RNase protection analysis using an antisense riboprobe specific for the SV40 component of the transgene (position of the riboprobe in indicated in Figure 4.2.1A). Three independent lines of MMTV/ILK-expressing mice (lines 2189, 363 and 1934) were identified using this approach, as indicated by the presence of a protected RNA fragment in the corresponding lanes of the RNase protection gel (Figure 4.2.1B).

4.2.2 MMTV/ILK expression results in precocious alveolar development and mammary gland hyperplasia

To determine if expression of the MMTV/ILK transgene resulted in an aberrant mammary gland phenotype, mammary gland wholemounts were prepared from virgin female MMTV/ILK mice. As shown in Figure 4.2.2, mild ductal hyperplasia was apparent in over 50% of these animals by the age of 6 months (Figure 4.2.2A). As compared to normal virgin FVB control glands (Figure 4.2.2B), the glands from MMTV/ILK mice consisted of an unusual number of secondary and tertiary branches, as well as small spiculated side buds normally seen during early to mid pregnancy. Histological sections of these glands revealed a multi-layered epithelium (Figure 4.2.2C, asterisk), harbouring an unusual number of abnormal mitotic structures (inset).

After 12 months of age, the phenotype of these mammary glands was more severe, consisting of an unusual number of well developed alveolar units, with tight

Figure 4.2.1 Targeted overexpression of ILK in the mouse mammary gland

(A) The 1.8kb EcoRI fragment of human ILK cDNA was placed downstream of the MMTV-LTR promoter/enhancer, in order to drive expression of ILK in the mouse mammary gland epithelium. The polyadenylation signal of SV40 (SV40 poly A) was included to ensure efficient processing of the RNA transcript. (B) Expression of the MMTV/ILK transgene was confirmed in 3 independent founder lines of transgenic mice, by ribonuclease (RNase) protection analysis of total mammary gland RNA. The riboprobe used in this analysis was generated against the transgene-specific SV40 sequence (position of the riboprobe is shown in A). An RNase protection riboprobe specific for the phosphoglycerate kinase (PGK) RNA message was used as an internal control for total RNA levels.

A


riboprobe
MMTV-LTR
ILK cDNA
SV40 poly A

B

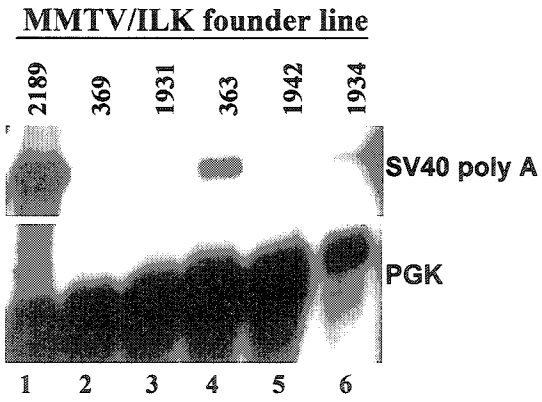
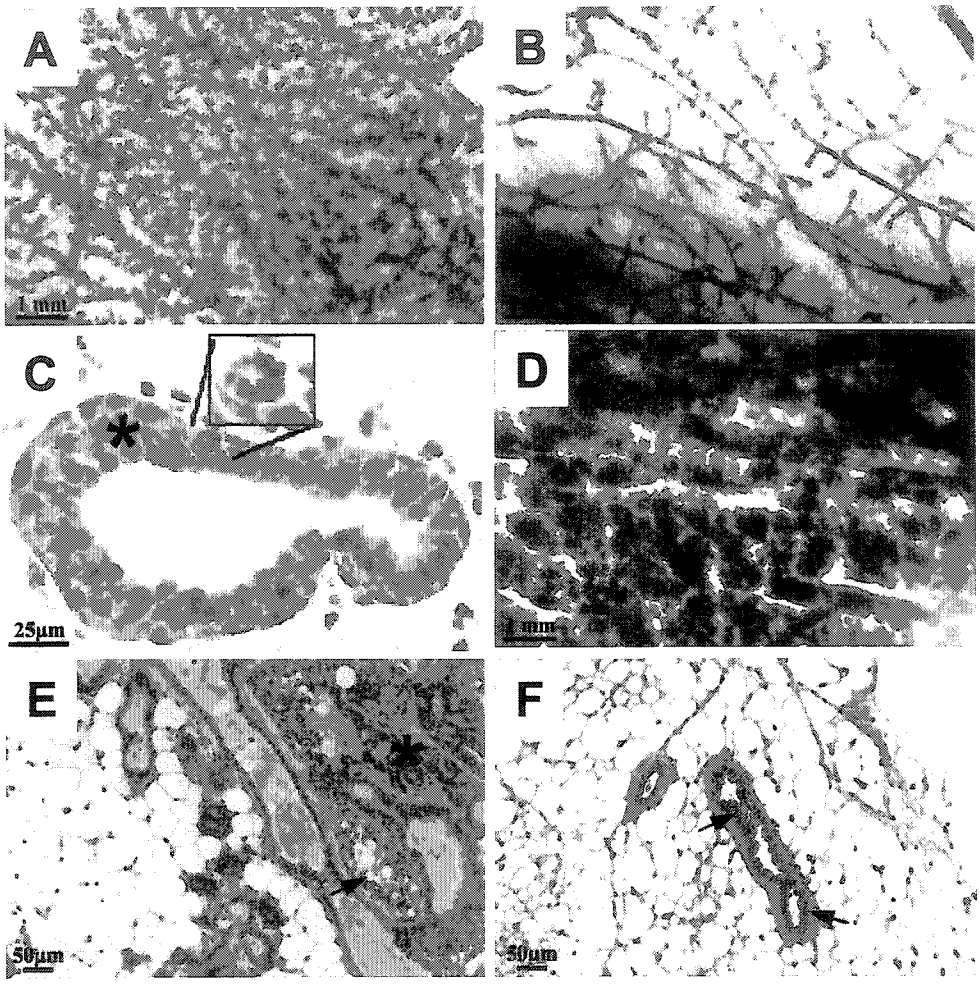

MMTV/ILK founder line
2189
369
1931
363
1942
1934
SV40 poly A
PGK
1
2
3
4
5
6

Figure 4.2.2 Mammary-specific expression of the MMTV/ILK transgene induces ductal hyperplasia and precocious alveolar development in virgin female transgenic mice

The impact of ILK overexpression in the mammary glands of MMTV/ILK mice was determined by examination of mammary gland wholemounts and sections. Mammary glands from 6 month-old virgin female MMTV/ILK mice exhibit mild ductal and acinar hyperplasia (**A**) when compared to glands from control FVB mice (**B**). A section of an MMTV/ILK-derived gland (**C**) reveals a multi-layered and aplastic ductal epithelium (indicated by the asterisk). The inset in panel C shows a cell harbouring an abnormal mitotic figure, which were common throughout these glands. By the age of 12 months, extensive lobulo-alveolar development was apparent in wholemounts from the MMTV/ILK animals (**D**), with evidence of epithelial hyperplasia (asterisk) and secretory vacuolization (arrow) clearly present within sections from these glands (**E**). Glands from 12 month-old FVB mice, by contrast, consist of single-layered luminal epithelia (arrows).


A
1 mm
B
C
*
25µm
D
1 mm
E
*
50µm
F
50µm

clusters resembling hyperplastic alveolar nodules (HANs) (Figures 4.2.2D and 4.2.2E, asterisk). In addition, secretory vacuolization was apparent in mammary gland sections from 12 month-old animals, a phenotype which is normally restricted to a lactating gland (Figure 4.2.2E, arrow). A mammary gland section from a 12 month-old virgin female control FVB mouse is shown for comparison in Figure 4.2.2F.

4.2.3 The hyperplastic mammary gland phenotype in MMTV/ILK mice is associated with the phosphorylation of downstream targets of ILK

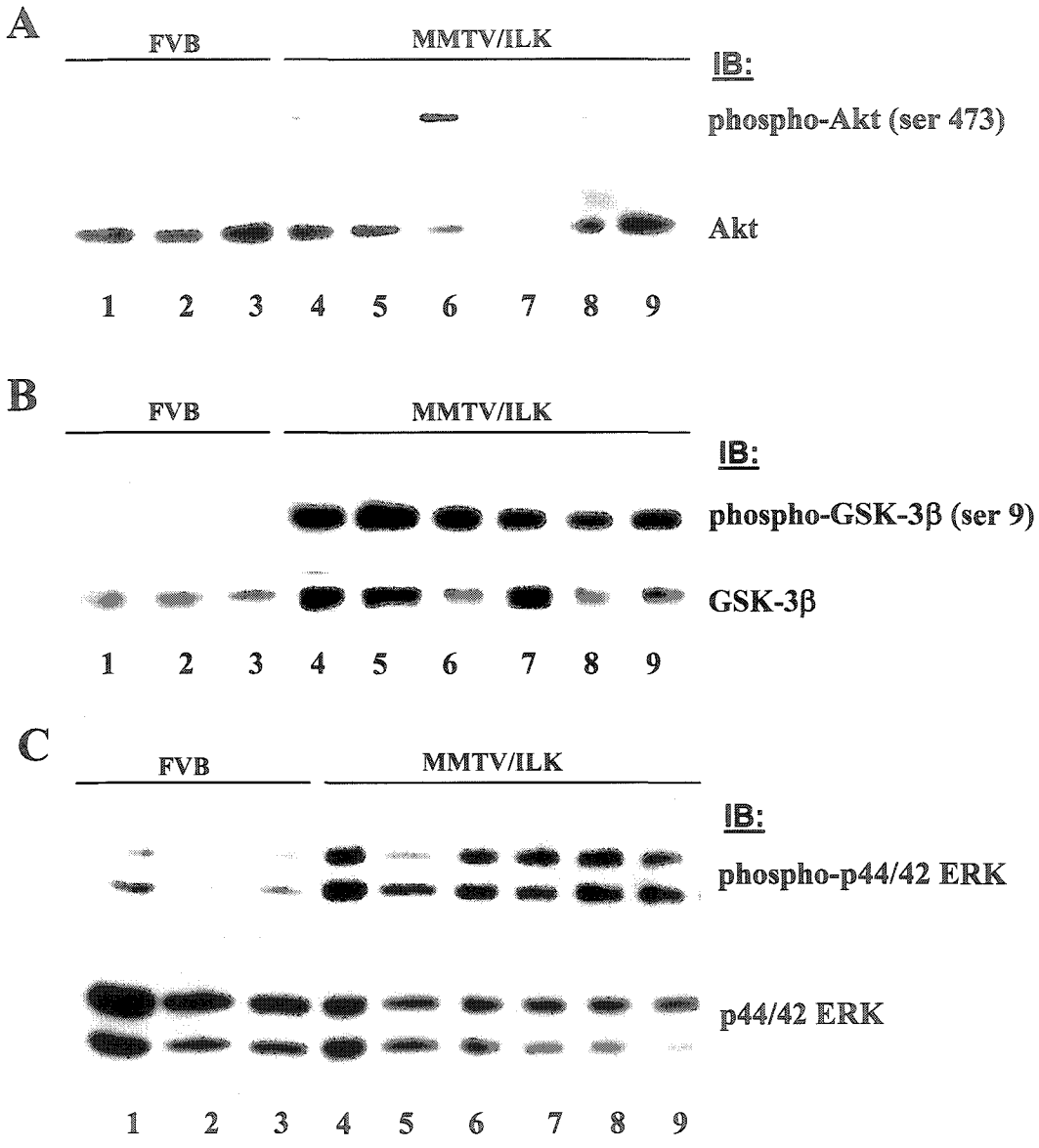
Given that PKB/Akt, GSK-3β and MAPK have been shown to be downstream targets of ILK kinase activity in cultured epithelial cells, the phosphorylation status of these proteins in the mammary glands of 6 month-old MMTV/ILK mice was examined by immunoblot analysis (Figure 4.2.3). The purpose of this analysis was to provide insight into the molecular mechanism behind the hyperplastic mammary gland phenotype in the MMTV/ILK mice.

The first target of ILK kinase activity to be examined in these mice was the anti-apoptotic protein PKB/Akt, which has been shown to be phosphorylated on serine residue 473 following ILK overexpression. To determine whether PKB/Akt was constitutively phosphorylated in response to MMTV/ILK expression, mammary tissue extracts from 6 month-old virgin female MMTV/ILK mice were subjected to immunoblot analysis using a phospho-specific antibody directed to serine 473 of PKB/Akt. As shown in Figure 4.2.3A, mammary glands derived from the MMTV/ILK mice were found to contain elevated levels of phosphorylated PKB/Akt protein, compared to glands from FVB control mice (compare lanes 4-9 with lanes 1-3). This difference was not due to changes in the levels of total PKB/Akt protein, as shown in the lower panel of Figure 4.2.3A.

The second important target of ILK kinase activity to be examined in these mice was the serine/threonine kinase GSK-3β, a negative regulator of the Wnt signaling pathway (Figure 4.2.3B). To determine whether GSK-3β was phosphorylated in the

Figure 4.2.3 Targets of ILK kinase activity are phosphorylated in mammary glands of MMTV/ILK mice

The hyperplastic mammary gland phenotype in 6 month-old MMTV/ILK mice is associated with the phosphorylation of molecules involved in the regulation of cell proliferation and survival. These include the anti-apoptotic PKB/Akt serine/threonine kinase (**A**), the Wnt pathway-associated GSK-3β (**B**), and p44/42 ERK (**C**). While phosphorylation of PKB/Akt and p44/42 ERK results in activation of these proteins, the phosphorylation of GSK-3β on serine 9 inhibits its negative regulatory role in the Wnt-β-catenin pathway.


A
FVB
MMTV/ILK
IB:
phospho-Akt (ser 473)
Akt
1 2 3 4 5 6 7 8 9
B
FVB
MMTV/ILK
IB:
phospho-GSK-3β (ser 9)
GSK-3β
1 2 3 4 5 6 7 8 9
C
FVB
MMTV/ILK
IB:
phospho-p44/42 ERK
p44/42 ERK
1 2 3 4 5 6 7 8 9

MMTV/ILK mice, the same tissue lysates were subjected to immunoblot analysis with a phopho-specific antibody directed to serine 9 of GSK-3β, a site known to inhibit GSK-3β kinase activity when phosphorylated. As with PKB/Akt, the results of this analysis showed that the phosphorylation of GSK-3β was elevated in the MMTV/ILK-derived mammary gland extracts, relative to those of control FVB mice (compare lanes 4-9 with lanes 1-3). Again, the increase in GSK-3β phosphorylation could not be attributed to differences in the levels of total GSK-3β protein (Figure 4.2.3B, lower panel).

The third important target to be examined using this approach was MAPK, another known target of ILK activity (Figure 4.2.3C). Consistent with the elevated phosphorylation of both PKB/Akt and GSK-3β, increased phosphorylation of MAPK was detected in the mammary gland extracts of 6 month-old virgin MMTV/ILK mice, relative to those of control mice (compare lanes 4-9 with lanes 1-3). The differences in the state of MAPK phosphorylation could again not be attributed to differences in the levels of total MAPK protein (Figure 4.2.3C, lower panel).

The results of these immunblot analyses, therefore, suggest that the concerted phosphorylation of these important cell signaling molecules may contribute to the induction of a hyperplastic phenotype in the mammary glands of MMTV/ILK mice.

4.2.4 Elevated expression of ILK predisposes the mammary epithelium to tumourigenesis

After 1 year of age, focal mammary tumours (Figure 4.2.4A) appeared in female mice from all 3 founder lines expressing the MMTV/ILK transgene (Table 4.2.4). In the best characterized strain (line 363), 34% of female animals developed focal mammary tumours with an average latency of 560 days (Table 4.2.4, Figure 4.2.4B). By contrast, no mammary tumours were observed in age-matched female FVB control mice (Table 4.2.4).

Figure 4.2.4 Overexpression of ILK in the mouse mammary epithelium results in the induction of mammary tumours

Focal mammary tumour (arrow in **A**) appeared in 35% of MMTV/ILK mice, after an average latency of 16 months (**B**).

A

B

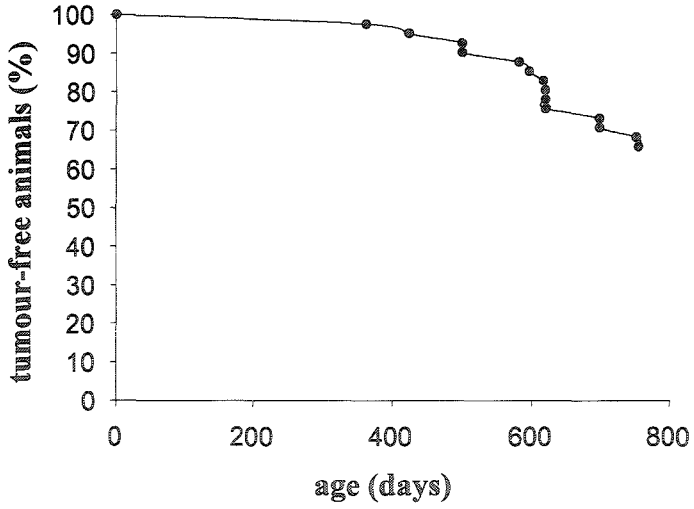

tumour-free animals (%)
100
90
80
70
60
50
40
30
20
10
0
0
200
400
600
800
age (days)

Table 4.2.4 Mammary tumour induction in MMTV/ILK transgenic mouse lines

Founder line	Tumour incidence	Tumour onset (average age)	Lung metastases	Tumour phenotype
ILK 363	14/41 (34%)	560 days (18.6 months)	3/14	adenocarcinoma (12*); spindle cell tumor (2)
ILK 2189	3/9 (33%)	440 days (14.7 months)	1/3	adenocarcinoma (1); spindle cell tumour (2)
ILK 1934	1/10 (10%)	364 days (12 months)	0/1	adenocarcinoma
FVB	0/21			N/A**

* adenocarcinomas show a degree of differentiation, from high to low, including mixed tumours containing both epithelial- and mesenchymal-like cell populations. The adenocarcinoma from ILK line 1934 was poorly differentiated. **An ovarian tumour appeared in 1 virgin FVB mouse at 20 months of age.

4.2.5 MMTV/ILK-derived tumours exhibit histological and molecular evidence of epithelial-to-mesenchymal transition (EMT)

Histological analysis of the MMTV/ILK-induced tumours revealed a somewhat diverse phenotype, ranging from well differentiated papillary adenocarcinomas (Figure 4.2.5.1A-D), to undifferentiated spindle cell tumours (Figure 4.2.5.1I-L). Several of the tumours consisted of differentiated epithelial cells interspersed within regions of mesencymal-like cell populations (Figure 4.2.5.1E-H), as indicated by the expression pattern of the epithelial markers cytokeratin-8 (CK8) and E-cadherin (Figure 4.2.5.1F and 4.2.5.1G), and the mesenchymal marker smooth muscle actin (SMA) (Figure 4.2.5.1H).

The presence of mesenchymal-like cell populations, particularly within tumours containing well defined glandular elements, therefore argues that tumourigenesis in the MMTV/ILK mice may involve an epithelial-to-mesenchymal transition (EMT). At the molecular level, the induction of EMT would be considered incompatible with the epithelial-specific MMTV promoter. As a result, expression of the MMTV/ILK transgene was measured in late-stage tumours from the MMTV/ILK mice, and compared to expression levels in adjacent regions of non-tumourigenic epithelium from the same mice. Consistent with the hypothesis of EMT, transgene expression in 9 of 11 tumours was found to be dramatically reduced in the MMTV/ILK animals, relative to adjacent gland (a representative comparison of matching tumour and adjacent tissue, for 3 animals, is shown in Figure 4.2.5.2A). Levels of total ILK protein, however, were found to be elevated in these tumours, as shown by both immunoblot (Figure 4.2.5.2B) and immunohistochemical (Figure 4.2.5.2C) analysis, using an anti-ILK antisera recognizing both the human and mouse isoforms. These results provide further evidence that tumourigenesis in MMTV/ILK mice indeed involves EMT.

Figure 4.2.5.1 Mammary tumours from MMTV/ILK mice exhibit histological evidence of an epithelial-to-mesenchymal transition

Three of the 14 MMTV/ILK-derived mammary tumours appeared as well differentiated adenocarcinomas (**A**), expressing the epithelial markers cytokeratin-8 (CK8) (**B**) and E-cadherin (**C**), with no evidence of a mesenchymal phenotype (**D**). Ten of the remaining tumours appeared less well differentiated (**E**), and were populated by a mixture of epithelial-like and non-epithelial-like cells (**F-H**). One tumour had a spindle cell morphology (**I**). This tumour failed to express the epithelial markers (**J, K**), yet expressed the smooth muscle actin (SMA) protein characteristic of mesenchymal-like cells (**L**).

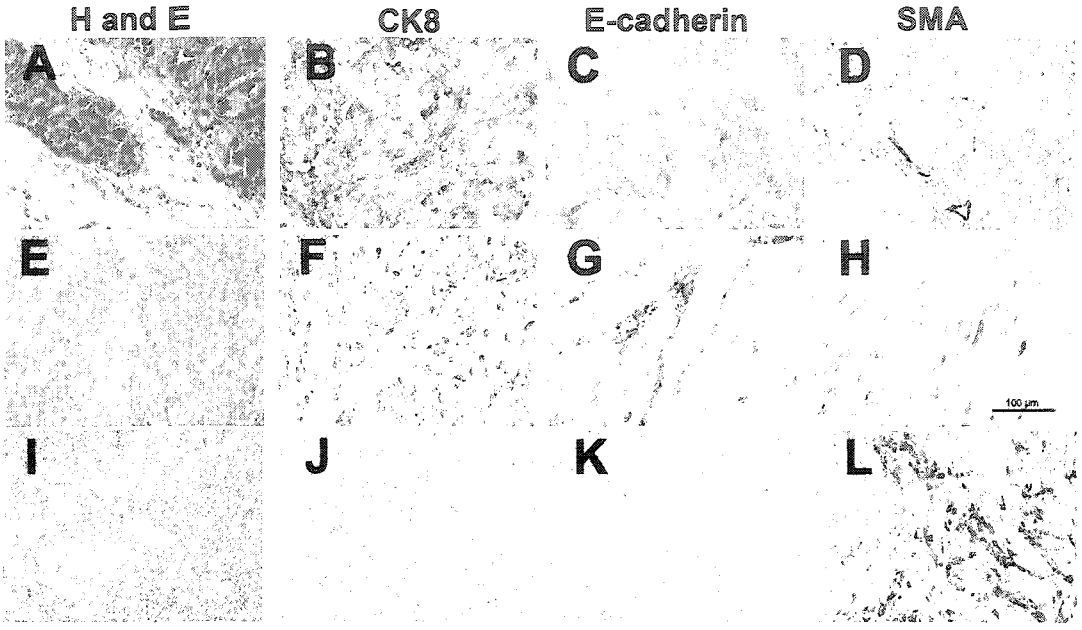
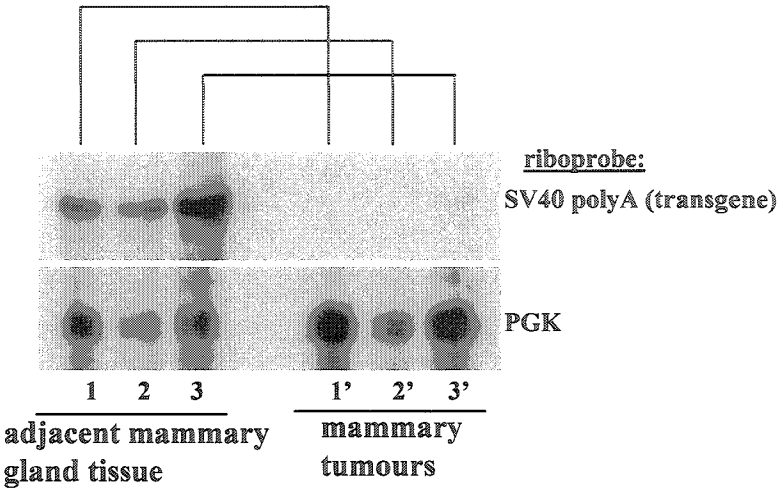

H and E
CK8
E-cadherin
SMA
A
B
C
D
E
F
G
H
I
J
K
L
100 µm

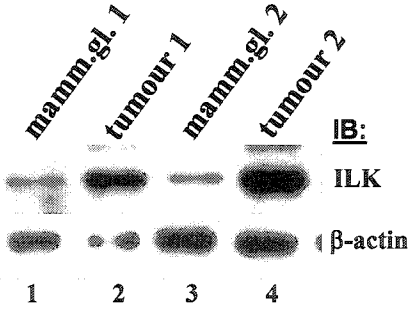
Figure 4.2.5.2 Endogenous ILK is upregulated in MMTV/ILK-derived tumours showing molecular evidence of EMT

(**A**) MMTV/ILK transgene expression in glands from 3 separate MMTV/ILK mice (lanes 1-3), as well as from tumours arising in those same animals (lane 1'-3'). Downregulation of MMTV/ILK transgene expression in these tumours suggests the induction of a genetic program incompatible with the epithelial-specific MMTV promoter. Despite the suppression of MMTV promoter activity in MMTV/ILK mice, tumours from these animals express higher levels of endogenous ILK protein (**B**, lanes 2 and 4), relative to adjacent mammary gland tissue (**B**, lanes 1 and 3). Subjecting MMTV/ILK-derived tumour tissue to immunohistochemical analysis with an anti-ILK antibody reveals expression of ILK protein throughout the tumour tissue (**C**).

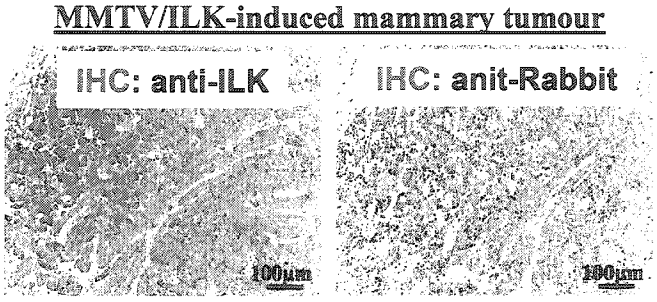
A


riboprobe:
SV40 polyA (transgene)
PGK
1 2 3
1' 2' 3'
adjacent mammary gland tissue
mammary tumours

B


mamm.gl. 1
tumour 1
mamm.gl. 2
tumour 2
IB:
ILK
β-actin
1 2 3 4

C


MMTV/ILK-induced mammary tumour
IHC: anti-ILK
IHC: anit-Rabbit
100µm
100µm

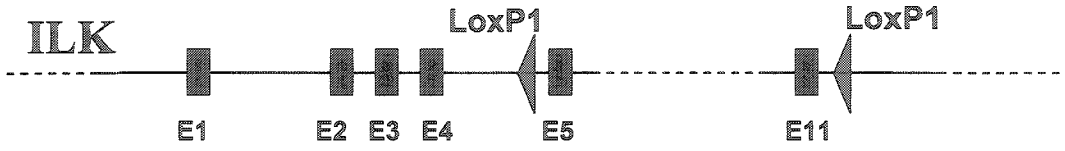
4.2.6 Expression of ILK is necessary for mammary gland development

The results presented in this Chapter demonstrate that ILK can contribute to mammary tumourigenesis when overexpressed in the mammary epithelium of transgenic mice. Whether expression of ILK is necessary for tumourigenesis induced by other oncogenes, such as PyV MT, requires further experimentation. One possible approach to this question would involve targeted deletion of a conditional allele of ILK in a mouse tumour model, similar to the experiment described in Chapter 3. In order to test the viability of this approach, mice were obtained in which exons 5 through 11 of the ILK gene were flanked by LoxP1 recombination sites (ILK^{LoxP1}) (Figure 4.2.6A). These mice were then crossed with the MMTV/Cre line described in Chapter 3, in order to obtain the appropriate genetic combinations. When compared to the mammary ductal outgrowth in 5.5 week-old control $ILK^{LoxP1/wt}$ MMTV/Cre mice, the extent of ductal arbourization and penetration into the fat pad was found to be impaired in age-matched animals of the $ILK^{LoxP1/LoxP1}$ MMTV/Cre combination (compare the 2 sets of glands from age-matched animals in Figure 4.2.6B). This result suggests that ILK, unlike β1-integrin (see Figure 3.2.2.1), may play an important role in development of the mouse mammary gland during puberty. As a result, analysis of tumourigenesis by ablation of ILK expression in a mouse tumour model will likely be complicated by developmental abnormalities. Determining the role of ILK in oncogene-induced tumourigenesis will therefore require an alternative experimental design, to be addressed at a later time.

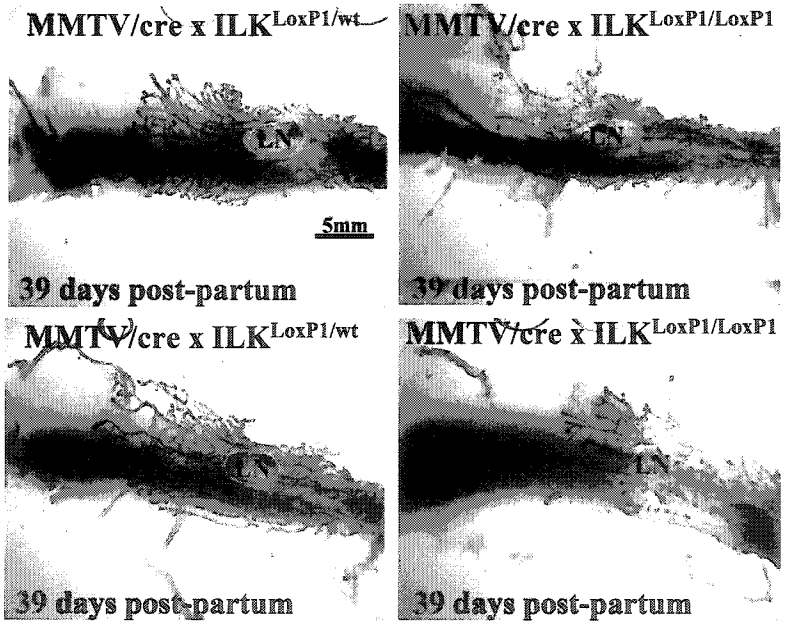
Figure 4.2.6 Expression of ILK is necessary for mammary gland development during puberty

Mice harbouring a LoxP1-flanked allele of ILK (ILK^{LoxP1}) (A) were crossed with the MMTV/Cre line, in order to determine if the expression of ILK was necessary for mammary gland development. When compared at 5.5 weeks of age (mid-point of maximum growth period), glands from $ILK^{LoxP1/LoxP1}$ MMTV/Cre mice showed evidence of impaired development, relative to control $ILK^{LoxP1/wt}$ MMTV/Cre-derived glands (B). Specifically, the developing glands from $ILK^{LoxP1/LoxP1}$ MMTV/Cre mice failed to migrate to the same extent into the fat pad (relative to the lymph node—LN), as compared to the control glands. In addition, the extent of ductal branching was dramatically reduced in the $ILK^{LoxP1/LoxP1}$ MMTV/Cre-derived glands, with evidence of fewer terminal end bud (TEB) structures.

A


ILK
LoxP1
LoxP1
E1
E2
E3
E4
E5
E11

B


MMTV/cre x ILK LoxP1/wt
LN
5mm
39 days post-partum
MMTV/cre x ILK LoxP1/LoxP1
LN
39 days post-partum
MMTV/cre x ILK LoxP1/wt
LN
39 days post-partum
MMTV/cre x ILK LoxP1/LoxP1
LN
39 days post-partum

4.3 DISCUSSION

4.3.1 ILK contributes to an oncogenic phenotype when overexpressed in the mouse mammary gland epithelium

The results presented in Chapter 4 confirm that the overexpression of ILK in the mouse mammary epithelium *in vivo* can induce a transformed phenotype and predispose the murine mammary gland to epithelial tumours. Although cultured mammary epithelial cells overexpressing ILK have been shown to undergo changes characteristic of oncogenic transformation (Yoganathan et al., 2002), mammary tumourigenesis in the MMTV/ILK mice confirms the oncogenic properties of ILK in a physiological context.

The phosphorylation of known downstream targets of ILK kinase activity in the mammary glands of MMTV/ILK mice provides a clue regarding the molecular mechanism behind mammary gland transformation in these animals. Given the focal nature of the MMTV/ILK-induced tumours, however, it is likely that additional genetic events are required during the transition from a hyperplastic gland to a solid mammary tumour. The activation of PKB/Akt, for example, has previously been shown to play a role in the oncogenic conversion of cultured epithelial cells (Chang et al., 2003; Mirza et al., 2000). The transformation of these cells by PKB/Akt involves the promotion of cell cycle progression, as well as the induction of anti-apoptotic pathways (Chang et al., 2003). In addition, maintenance of the cellular translational machinery through regulation of the mTOR pathway has also been shown to contribute to PKB/Akt-induced transformation (Aoki et al., 2001). Mice expressing activated alleles of PKB/Akt in the mammary epithelium, however, fail to develop mammary gland tumours, despite an early mammary gland phenotype resembling the precocious alveolar development seen in the MMTV/ILK animals (Hutchinson et al., 2001; Schwertfeger et al., 2001). Expression of activated PKB/Akt in the mammary glands of the MMTV/erbB2(neu) model, however,

resulted in a dramatic increase in the rate of tumourigenesis in these animals (Hutchinson et al., 2004).

The MMTV/ILK mice might therefore represent a new model of multi-step tumourigenesis, which more accurately reflects the nature of malignant disease progression in humans. Other models found in this category include the MMTV/c-myc (Sinn et al., 1987) and MMTV/cyclin D1 strains (Wang et al., 1994), as well as a strain expressing the casein kinase 2 alpha (CK2α) gene, also under transcriptional control of the MMTV-LTR (Landesman-Bollag et al., 2001). In addition, this category includes those mice expressing the wild-type erbB2 allele (Guy et al., 1992b), as well as an activated erbB2 allele targeted to the endogenous erbB2 promoter (Andrechek et al., 2000). Interestingly, two of these models (MMTV/cyclin D1 and MMTV/CK2α) exhibit the same tumour kinetics as the MMTV/ILK mice, with focal mammary tumours appearing in the animals at an average age of 18 months (Landesman-Bollag et al., 2001; Wang et al., 1994). Since ILK and CK2α are both involved in regulating the Wnt signaling pathway upstream of cyclin D1 (D'Amico et al., 2000; Song et al., 2003; Song et al., 2000), it is tempting to speculate that the similarity in tumour phenotypes is due to overlapping mechanisms of tumour induction in these animals, perhaps involving cell cycle progression.

Tumours from the MMTV/ILK and MMTV/CK2α strains are also comparable with regards to phenotype, in both cases showing histological evidence of EMT (Landesman-Bollag et al. (2001) and Figure 4.2.5.1). In addition, the MMTV promoter was reported to be silenced in the CK2α-derived tumours, similar to that observed in the MMTV/ILK model (Landesman-Bollag et al., 2001). As discussed in section 4.2.5, this latter phenomenon implies the induction of a mesenchymal-like genetic program in these tumours, incompatible with the epithelial-specific MMTV-LTR. In both cases, however, silencing of the MMTV-driven transgene was compensated by a corresponding upregulation in expression levels of the endogenous protein (Landesman-Bollag et al. (2001) and Figure 4.2.5.2). This observation suggests that there is an obligatory need to maintain elevated levels of ILK and CK2α protein expression during mammary gland

tumourigenesis. With regards to the MMTV/ILK model, this phenomenon provides an indication as to the potential importance of ILK in the tumourigenic process. In addition, the compensatory upregulation of ILK may represent another genetic event contributing to the long latency of tumour induction in these animals.

Prior to the onset of this work, ILK was found to be overexpressed in neuroectodermal tumours and human melanoma cell lines (Chung et al., 1998; Janji et al., 1999). Since this work was initiated, however, numerous examples of ILK overexpression in human epithelial cancers have been cited (Ahmed et al., 2003; Dai et al., 2003; Graff et al., 2001; Marotta et al., 2001; Yoganathan et al., 2002). Importantly, the overexpression of ILK in almost all of these tumour samples was found to be associated with progression to a more malignant phenotype (Ahmed et al., 2003; Dai et al., 2003; Graff et al., 2001; Yoganathan et al., 2002). The induction of mesenchymal-like tumours in the MMTV/ILK mice, therefore, provides experimental confirmation that ILK may be playing a causative role in promoting disease progression in human breast cancer patients. Indeed, the therapeutic potential of ILK as a drug target has been confirmed by a recent report describing the use of a small molecule inhibitor of ILK kinase activity to successfully block the growth of prostate tumours in a murine xenograft model (Tan et al., 2004).

4.3.2 Expression of ILK is required for normal mammary gland development during puberty

Mice expressing the MMTV/Cre transgene, and harbouring two copies of a LoxP1-flanked ILK (ILK^{LoxP1}) allele, exhibited evidence of impaired mammary gland development during puberty (see Figure 4.2.6). The glands of these mice were examined at 39 days post-partum, which represents approximately the mid-point of a rapid developmental growth period. When compared to normal FVB control mice, glands from the $ILK^{LoxP1/LoxP1}$ MMTV/Cre combination were disorganized with regards to the growth pattern of the mammary tree, and had a reduced number of terminal end bud (TEB)

structures. In addition, the glands from the $ILK^{LoxP1/LoxP1}$ MMTV/Cre animals failed to migrate into the mammary gland fat pad to the same extent as those of control mice.

This result provides the first experimental demonstration that ILK may play a role in epithelial physiology *in vivo*. Previous experiments involving targeted ablation of ILK in mouse models have focused on tissues of mesenchymal origin, specifically chondrocytes (Grashoff et al., 2003; Terpstra et al., 2003). Any other experiments designed to address the role of ILK *in vivo* have been performed in invertebrates, involving the analysis of natural mutations in ILK function in flies and worms (Mackinnon et al., 2002; Zervas et al., 2001). Using the drosophila and *C.elegans* model systems, these authors showed that ILK function is critical for the assembly of integrin-mediated muscle attachment sites, revealing a role primarily in tissues of mesenchymal origin. A putative mesenchymal role for ILK is consistent with the expression pattern of ILK in mouse and humans, which is markedly higher in skeletal and heart muscle, as compared to other tissues (Hannigan et al., 1996; Li et al., 1997b).

The mammary gland phenotype in $ILK^{LoxP1/LoxP1}$ MMTV/Cre mice therefore reveals a novel role for ILK in epithelial physiology. The impaired migrational capacity and disorganized growth pattern of the $ILK^{LoxP1/LoxP1}$ MMTV/Cre-derived glands suggests that ILK may be necessary for some aspect of precursor cell proliferation or survival in the TEBs. It is conceivable that ILK-induced activation of PKB/Akt or cyclin D1 expression, for example, may be required to maintain growth of the cells occupying the leading edge or 'cap' structure of the TEBs. Interestingly, this phenotype was not observed following mammary-specific ablation of β1-integrin expression (see Figure 3.2.2.1). This discrepancy was unexpected, since the muscle attachment phenotype described in ILK-null flies and worms was a phenocopy of β1-integrin mutations in those organisms (Mackinnon et al., 2002; Zervas et al., 2001).

The impaired mammary gland phenotype in $ILK^{LoxP1/LoxP1}$ MMTV/Cre mice therefore suggests that ILK plays a more pleiotropic role than β1-integrin in the mammary gland epithelium. Since ILK kinase activity can be induced by growth factor receptor activation of PI3-K in human epithelial cells (Delcommenne et al., 1998), the

requirement for ILK during mammary gland development may reflect its role in growth factor receptor signaling, for example. Alternatively, the ILK and β1-integrin-null phenotypes in mice versus flies and worms may reflect the way in which ILK is activated in different tissues. There is experimental evidence, for example, that ILK plays a role exclusively as a structural scaffolding protein in the muscle of drosophila and *C.elegans*, since the null-phenotype can be rescued by expression of a kinase-dead allele of ILK (Mackinnon et al., 2002; Zervas et al., 2001). In the case of human and mouse epithelial cells, however, the kinase activity of ILK is believed to play the most important role in regulating the phenotypic properties of the cells. Therefore, 'activating' the ILK molecule itself may be a concept which is fundamentally different in the two tissue types, particularly since there is evidence that the kinase aspect of ILK-mediated signaling can also be induced by growth factor receptors. Whereas other receptor types may serve as surrogates for ILK activation in β1-integrin-null epithelial cells, it is conceivable that the physical association with β1-integrin is necessary for facilitating the scaffolding function of ILK at muscle attachment sites in the invertebrates. After comparing the β1-integrin and ILK-null phenotypes in the mammary glands of mice with the muscle phenotype described in flies and worms, it is clear that much has yet to be learned regarding the biological relationship between these two molecules.

CHAPTER 5

Summary and Future Directions

5.1 Determining the role of β1-integrin signaling in mammary gland tumourigenesis

The experimental results presented in Chapters 3 and 4 of this thesis demonstrate two important principles regarding the role of β1-integrin signaling in mammary gland tumourigenesis. The first of these principles concerns the role of the β1-integrin protein itself, where target ablation of β1-integrin expression was shown to result in the inhibition of tumourigenesis in a transgenic mouse model of human breast cancer. These results, as presented in Chapter 3, provide a convincing demonstration that β1-integrin expression is required for the induction of mammary tumourigenesis *in vivo*. Since these experiments were performed in a physiologically relevant context, the results presented in Chapter 3 suggest that the expression pattern of β1-integrin in advanced human cancers (Shaw, 1999) may indeed be clinically relevant. Importantly, the proliferative block observed in AdCre-infected $\beta1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumour cells *in vivo* (see Figure 3.2.6.2) resembles the tumour cell dormancy induced by inhibition of FAK signaling in human carcinoma cells (Aguirre Ghiso, 2002; Liu et al., 2002). When considered together, these results suggest that β1-integrin-mediated FAK activation may be involved in regulating the progression of malignant disease in human cancer patients.

The second major principle regarding β1-integrin signaling in mammary tumourigenesis was demonstrated in Chapter 4. Through the establishment of transgenic mice overexpressing the β1-integrin-associated ILK in the mammary gland epithelium, it was confirmed *in vivo* that ILK may play a direct role in the induction and progression of epithelial cancers. Since the overexpression of ILK has also been reported in advanced human cancers (Persad and Dedhar, 2003), the induction of tumours in the MMTV/ILK

mice may again be clinically relevant to the initiation and progression of human breast cancer. The recent use of a small molecule inhibitor of ILK kinase activity to block the growth of a prostate cancer xenograft indeed provides a convincing demonstration that ILK may represent a viable target for the treatment of human cancers (Tan et al., 2004).

Given that ILK is an effector of β1-integrin signaling, the tumourigenic phenotype in the MMTV/ILK mice provides an important demonstration of the pathological consequences resulting from deregulated β1-integrin signaling. Although integrins themselves have not been traditionally regarded as oncogenes, there is evidence that the deregulation of their associated intracellular signaling pathways, such as through ILK or FAK activation, is an important contributing factor to the transformation process. The results in Chapter 4 provide an important illustration of this principle.

5.2 The physiological requirement for β1-integrin signaling in mammary tumourigenesis: role of the tumour stroma

The conversion of a primary epithelial cell to a malignant tumour has historically been attributed to oncogenic events within the cell, such as mutations in growth-promoting pathways and inactivation of tumour suppressor genes and gene products (Hanahan and Weinberg, 2000). Evidence is accumulating, however, that the tumourigenic process may depend as much on the extracellular context of the tumour cell, as on the genetic program (Bissell et al., 2002; Ronnov-Jessen et al., 1996; Shekhar et al., 2003; Wiseman and Werb, 2002).

A role for the extracellular matrix during tumourigenesis was first suggested by descriptions of a "reactive tumour stroma," a phenomenon in which tumours accumulate dense regions of an ECM composed primarily of fibrous proteins such as collagen. Interestingly, it was recognized that the accumulation of stroma in tumours in many ways resembled the process of wound healing, suggesting that this event represented a specific physiological response (Dvorak, 1986). The presence of a reactive tumour stroma has

since been described in mammary tumours of transgenic mouse models, as well as in human mammary carcinomas (Ronnov-Jessen et al., 1996; Wiseman and Werb, 2002).

Stromal deposition in human breast cancer may indeed have important clinical implications, since it is correlated with a more aggressive disease state (Ronnov-Jessen et al., 1996; Shekhar et al., 2001). Interestingly, breast tissue density is considered an important predictive indicator of breast cancer in humans (Boyd et al., 1998). Predisposition of mammary carcinoma in individuals with a relative high breast density suggests that the presence of a dense breast stroma may indeed play a supportive or even causative role in the etiology of human breast cancer. Indeed, a tumour-promoting role for the stroma has been demonstrated experimentally in a transgenic mouse model overexpressing the matrix metalloproteinase stromelysin-1 (MMP-3) in the mammary gland (Sternlicht et al., 2000). In this case, transformation of the mammary gland epithelium was induced *in vivo* by the MMP-3-mediated remodeling of the ECM.

There are several possible explanations for the stromal presence during tumourigenesis. First, the stroma provides a rapid source of sequestered growth factors which may facilitate the growth of the tumour (Pupa et al., 2002). Second, the stroma may provide a structural platform to support a tumour vasculature, which is known to be a required element of tumourigenesis (Folkman, 2002). Third, the fibrous ECM components may provide survival and growth-promoting signals directly to the transformed epithelial cells, through adhesive events mediated by the integrin receptors.

Of the three potential roles of the tumour stroma, the last is the most compelling with regards to the transformation of the epithelium by activated growth factor receptors, particularly during the early stages of tumourigenesis. As discussed throughout this thesis, signaling through the integrin receptors provides a degree of molecular cross talk which may be necessary for the optimal activation of growth-promoting pathways. The inhibition of tumourigenesis in the $\beta1^{LoxP1/LoxP1}$MMTV/Cre/PyV MT mice, therefore, may be due to the absence of BM-derived signals, rather than the loss of β1-integrin protein *per se*. Similarly, tumourigenesis in the MMTV/ILK mice may reflect the elevated and constitutive delivery of signals which would otherwise be provided by

engagement of adhesion receptors with BM-derived ligands. With regards to the MMTV/ILK model, it is interesting to point out that the latency of tumour induction, as well as the penetrance and histological description of tumour phenotype in these mice, is identical to that of the MMP-3-expressing model described above (Sternlicht et al., 2000).

Although suggestions for a tumourigenic role for the stroma are based primarily on descriptive evidence, the results of a recent experiment have revealed a direct link between breast tissue density and FAK-mediated cell cycle progression (Wozniak et al., 2003). In this experiment, human breast epithelial cells were plated in a dense collagen matrix resembling the dense human breast stroma reported in high-risk breast cancer patients (Boyd et al., 1998). In this environment, the mammary epithelial cells grew in a disorganized pattern, failing to form the alveolar and tubular structures normally seen in a looser, more physiologically normal matrix (Wozniak et al., 2003). Inhibition of FAK phosphorylation, however, locked the cells in a differentiated state, rendering them resistant to the irregular growth characteristics otherwise induced by the dense extracellular environment. Consistent with the results of earlier experiments (Honda et al., 1998; Zhao et al., 1998), the suppression of proliferation in these cells was associated with inhibition of FAK phosphorylation specifically on the c-Src-binding site, tyrosine 397 (Wozniak et al., 2003). It is conceivable, therefore, that the suppression of FAK phosphorylation in Cre-expressing cells renders those cells resistant to tumourigenesis in the $\beta1^{LoxP1/LoxP1}$MMTV/Cre/PyV MT mice, through the induction of a non-proliferative, differentiated phenotype.

5.3 An alternative role for the β1-integrin subunit in mammary gland tumourigenesis: a signaling co-factor for the uPA receptor

Another interesting and important example of the way in which the β1-integrin subunit mediates interactions with the extracellular environment involves the physical association between β1-integrin and the urokinase plasminogen activator (uPA) receptor

(Wei et al., 1996). Binding of uPA to its receptor results in an enzymatically active uPA molecule, which catalyzes the proteolytic conversion of plasminogen to the serine protease plasmin. By degrading fibrin-rich structures within wounds and blood clots, as well as facilitating MMP-mediated degradation of ECM components, the uPA-uPAR system plays an important role in tissue remodelling during wound healing and migration. In addition, plasmin promotes the proteolytic activation of latent growth factors, such as TGF-β, sequestered within the extracellular stroma (Lyons et al., 1990).

Both uPA and its receptor have been found to be overexpressed in human cancers, including those of the breast (Duffy and O'Grady, 1984; Duffy et al., 1990; Pyke et al., 1991; Romer et al., 1994). The expression of these molecules along the invasive front of a tumour likely reflects a role in promoting the invasive properties of the tumour, through degradation of the underlying basement membrane. (Exploiting the tissue remodeling role of uPA in this context provides an interesting demonstration of the concept that tumours resemble 'wounds that do not heal' (Dvorak, 1986)). In addition, there is experimental evidence that the uPA receptor is involved in the activation of intracellular signaling pathways which promote proliferation of the tumour cells.

Recent experiments designed to address the contribution of the uPA receptor in tumour cell proliferation have revealed an important role for the β1-integrin subunit in this process. For example, inhibition of uPAR/β1-integrin complexes suppresses the growth of colon and hepatocellular carcinoma cells *in vivo*, through the inhibition of ERK activation (Aguirre Ghiso et al., 1999; van der Pluijm et al., 2001; Yu et al., 1997). Similarly, inhibition of uPAR/β1-integrin complex formation blocked the growth of human breast cancer cells in a xenograft model of human breast cancer (Aguirre Ghiso, 2002). Since the induction of dormancy in these human carcinoma cell lines was found to be associated with a downregulation of FAK phosphorylation and FAK-mediated proliferation (Liu et al., 2002), these results provide additional insight into the inhibition of tumourigenesis in the $\beta1^{LoxP1/LoxP1}$MMTV/Cre/PyV MT mice. In addition, the results of these experiments demonstrate the biological importance of lateral receptor cross talk in promoting tumour growth. The nature of this cross talk, however, was found to go

beyond that of just the uPAR/β1-integrin complex, since the uPAR/β1-integrin-mediated activation of FAK was shown to regulate the EGFR-mediated proliferation of the tumour cells *in vivo* (Liu et al., 2002). Combined with the results presented in Chapter 3 of this thesis, these results have important implications regarding the role of β1-integrin expression during the growth of human tumours induced by activated growth factor receptors.

5.4 Contributions from other integrin receptors: taking the β4-integrin subunit into consideration

Given the hypothesized role of the tumour stroma during cancer progression, combined with the physiological and signaling roles of the integrin adhesion receptors, the past decade has seen a great deal of interest into the integrin contribution to the pathological state. With regards to human breast cancer, the focus has been limited to the pathological roles of receptors containing the β1 and β4-integrin subunits, which are the only β-integrin subunits known to be expressed in this tissue (Bouvard et al., 2001; Fassler and Meyer, 1995). Much of what has been learned about the pathological roles of these subunits, in addition to their role in normal mammary gland development, has been contributed indirectly through the analysis of clinical specimens and the manipulation of cultured mammary epithelial cells. Again, elucidating the physiological roles of β1 and β4 integrins directly *in vivo* has been limited by the embryonic lethality of targeted deletions in these genes (Shaw, 1999; Zutter et al., 1998).

Integrin receptors containing both the β1 and β4 integrin subunits have been found to be expressed in advanced human breast carcinomas, as shown by RNA expression analysis and immunohistochemical techniques (Weaver et al., 2002; Weaver et al., 1997). The potential clinical significance of these expression patterns was subsequently demonstrated at the cellular level, through the application of molecular techniques designed to block expression or activity of the β1- and β4-containing heterodimers in cultured human breast cancer cell lines (Wang et al., 2002; Weaver et al.,

2002; Wewer et al., 1997). Depending on the particular experiment, the inhibition of these receptors resulted in either apoptotic death of the cells (Wang et al., 2002; Weaver et al., 1997), inhibition of proliferation (Wang et al., 2002; Weaver et al., 2002; Weaver et al., 1997; Wewer et al., 1997), or suppression of the tumourigenic phenotype in nude mice (Weaver et al., 1997). When considered together, the results of these experiments strongly suggested that integrin receptors containing either the β1 and β4 subunits are involved in mediating the malignant phenotype of human breast cancer.

The role of the β1-integrin subunit during tumourigenesis *in vivo* was confirmed in the experiments presented in Chapter 3 of this thesis. Consistent with the results of the earlier cell culture-based experiments (Weaver et al., 2002), the results presented in Chapter 3 indicate that β1-integrin expression is required primarily for maintaining the proliferative capacity of oncogene-induced tumour cells. The β4-integrin subunit, by contrast, has been shown to play an important role in protecting against apoptotic cell death in cultured human breast cancer cells . It is conceivable, therefore, that the β1 and β4-integrin-containing receptors perform different, yet complimentary roles during tumourigenesis and disease progression. In this regard, it will be interesting to determine if expression of the β4 subunit is providing protection against cell death in the β1-null $\beta1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT-derived tumour cells, both *in vitro* and *in vivo*. Similarly, it is conceivable that a contribution from the β4-integrin subunit might support normal mammary gland development in the $\beta1^{LoxP1/LoxP1}$ MMTV/Cre animals. Targeted ablation of the β4-integrin subunit in the MMTV/PyV MT model would be a good approach to begin addressing these issues.

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